The Role of Airway Epithelia in Anti-pathogen Responses, Innate Immunity and Lung Repair

Teresa McCabe

University College London

and

The Francis Crick Institute

PhD Supervisor: Andreas Wack

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Declaration

I Teresa McCabe confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Airway epithelia are the first targets of influenza A virus (IAV) infection and the first cells to respond, contributing to immunity, pathology and recovery. The magnitude and quality of epithelial responses may depend on exacerbations like bacterial superinfections and be determined by the host genetic background. We therefore use a primary mouse tracheal epithelial cell (mTECs) cultures to identify epithelial determinants of susceptibility and protection.

We assessed mTEC responses to IAV-bacterial co-exposure and found that the massive *in vivo* cytokine and chemokine response to co-infection is reflected *in vitro* by strongly increased epithelial responses to a combined viral-bacterial stimulus compared to single stimuli. The antiviral transcriptional responses dominate the overall response *in vivo* and *in vitro*.

We also compared the epithelial response to IAV between high interferon producing, susceptible 129S8 mice, and IAV-resistant moderate interferon producers, C57BL/6 mice. We found that 129S8-derived mTECs do not produce more interferons in response to IAV but respond more strongly to interferons and IAV by induction of cytokines and interferon-stimulated genes. 129S8-derived epithelia also proliferate and differentiate less well than C57BL/6 mTECs, suggesting reduced repair potential.

The initial epithelial cell-intrinsic antiviral response and control of IAV depends on type I and/or III interferons (IFNαβ or IFNλ). When IFNα and IFNλ influenza treatments were compared, IFNα stimulated both innate immune cells and mTECs, increasing immunopathology and mortality. IFNλ treatment only induced antiviral epithelial responses but not the immune-mediated pathology

triggered by IFNα. Therefore, IFNλ treatment helps control IAV and protects without inducing immunopathology.

During recovery, lung epithelial stem cells must proliferate and differentiate to repair infection-induced epithelial damage. We found that IFN impaired epithelial regeneration by reducing stem cell proliferation and differentiation, likely through IFN-induced blockade of epidermal growth factor signalling. Thus antiviral immune responses, if mistimed or excessive, may impede post-infection lung repair.

Together, mTECs help identify determinants of susceptibility and protection.

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“Education is not the filling of a pail, but the lighting of a fire”

William Butler Yeats

No truer has a quote reflected these last 4 years of my PhD. I entered into the NIMR/FCI with a burgeoning flame and have emerged with a roaring bonfire. There are many people that I owe my sincere and wholehearted thanks to for building and nurturing that fire.

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Abbreviations

129	129S8 or 129SvEv mice
ADCC	Antibody-dependent cell-mediated cytotoxicity
AEC	Airway epithelial cell
AHR	Aryl hydrocarbon receptor
ALI	Air liquid interface
AMP	Antimicrobial peptide
APC	Antigen-presenting cell
B6	C57BL/6
BAL	Bronchoalveolar lavage
CbpA	Choline-binding protein A
Ccdc67	Coiled-Coil Domain-Containing Protein 67
CCL2	Chemokine (C-C motif) ligand 2
Ccno	Cyclin O
CCSP	Club cell secretory protein
CD133	Prominin-1
CD200	OX-2 membrane glycoprotein
CD200R	OX-2 membrane glycoprotein receptor
CD49f	Integrin alpha 6
CD66a	Ceacam1
cDNA	complementary DNA
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony-forming unit
ChoP	Phosphorylcholine
COPD	Chronic obstructive pulmonary disease
cRNA	complementary RNA
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
CXCL1	Chemokine (C-X-C motif) ligand 1
d.p.i.	Days post infection
D39	Streptococcus pneumoniae D39 (virulent serotype 2)
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DR5	Death receptor 5
dsRNA	double stranded RNA
EC50	Half maximal effective concentration
ECMV	Encephalomyocarditis virus
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EREG	Epiregulin
FCI-MH	Francis Crick Institute-Mill Hill
FGF	Fibroblast growth factor
FGF-10	Fibroblast growth factor 10
Foxj1	Forkhead box protein J1
GM-CSF	Granulocyte macrophage colony-stimulating factor
H1N1	Influenza A virus subtype H1N1
H3N1	Influenza A virus subtype H3N1
H5N1	Influenza A virus subtype H5N1
HA	Hemagglutinin
HCL	Hairy cell leukaemia
HGF	Hepatocyte growth factor
HIV-1	Human immunodeficiency virus-1
HK	Heat-killed
HLA	Human leukocyte antigen
HPAI	Asian highly pathogenic avian influenza
Hprt1	Hypoxanthine Phosphoribosyltransferase 1
HSV-2	Herpes simplex virus-2
IAV	Influenza A virus
IDO	Indoleamine 2,3-dioxygenase
ifi203	Interferon activated gene 203
IFITM3	Interferon-induced transmembrane protein 3
IFN	Interferon
IFNAR	Interferon alpha/beta receptor
IFNλR	Interferon lambda receptor
IgG	Immunoglobulin G
IL	Interleukin
IL-10R2	Interleukin 10 receptor subunit beta
IL-28 (A/B)	Interleukin-28 (A/B)
ILD	Interstitial lung disease
iNOS	Nitric oxide synthases
IPA	Ingenuity pathway analysis
IPF	Idiopathic pulmonary fibrosis
IRF	Interferon regulatory factors
ISG	Interferon stimulated genes
JAK	Janus kinase
KGF	Keratinocyte growth factor
Krt5	Keratin 5
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
Mcidas	Multiciliate Differentiation And DNA Synthesis Associated Cell Cycle Protein

MCMV	Mouse cytomegalovirus
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MSV	Mouse Sarcoma virus
mTEC	mouse tracheal epithelial cell
MUC5AC	Mucin 5AC
MUC5B	Mucin 5B
Mx	Interferon-induced GTP-binding protein
MyD88	Myeloid differentiation primary response 88
NA	Neuraminidase
NEP	Nuclear export protein
NFκB	Nuclear factor-κB
NGFR	Nerve growth factor receptor
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain-like receptors
NP	Nucleoprotein
OAS	2'-5' Oligoadenylate Synthetase
OD	Optical Density
p-EGFR	phosphorylated-epidermal growth factor receptor
p63	Transformation-related protein 63
PA	Polymerase acidic protein
PAFR	Platelet activating-factor receptor
Pam3CSK4	N-Palmitoyl-S-3Cys-Ser-(Lys)4-3HCL
PAMPs	Pathogen associated molecular patterns
PB1	Polymerase basic protein 1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBS2	Polymerase basic protein 2
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PR8	Influenza A virus (strain A/Puerto Rico/8/1934 H1N1)
PRR	Pattern recognition receptor
PsaA	Pneumococcal surface adhesion A
QTL	Quantitative trait locus
RIG-I	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
RNaseL	2'-5' oligoadenylate synthetase-dependent ribonuclease
RPAIN	RPA Interacting Protein
Rsad2	Radical S-adenosyl methionine domain containing 2/Viperin
RT-qPCR	Quantitative reverse transcription PCR
S100a	S100 calcium-binding protein A
S100a8	Calgranulin A
S100a9	Calganulin B

SA	Sialic acid
SAR	Secondary attack rate
Scgb1a1	Secretoglobin family 1A member 1
SEM	Standard error of the mean
Slpi	Secretory Leukocyte Peptidase Inhibitor
SNP	Single nucleotide polymorphism
SPC	Surfactant-associated protein
STAT	Signal transducer and activator of transcription
SV40	Simian vacuolating virus 40
T-EGFR	Total-epidermal growth factor receptor
TCID50	50% cell culture infectious dose
TEER	Transepithelial electrical resistance
TGF	Transforming growth factor
Th1	T helper 1 cells
Th2	T helper 2 cells
TIGR4	Streptococcus pneumoniae TIGR4 (virulent serotype 4)
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFSF10	Tumor necrosis factor superfamily member 10
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
TRIM25	Tripartite-motif-containing protein 25
TYK	Non-receptor tyrosine-protein kinase
VEGF	Vascular endothelial growth factor A
Veh Ctrl	Vehicle control
vRNA	viral RNA
vRNP	viral ribonucleoprotein
WHO	World health organisation
Wt	Wildtype
X31	Influenza A virus (strain A/HongKong/1/68 (H3N2))

Chapter 1. Introduction

1.1 Respiratory infections

1.1.1 Influenza A Virus

Viral genome and classification

The influenza virus was first isolated in 1933 by Smith, Andrewes and Laidlaw (Smith *et al.* 1933). Influenza viruses belong to the Orthomyxoviridea family of enveloped negative-sense RNA viruses. The five genera in this family include influenza types A, B, and C, isavirus (infectious salmon anemia virus) and thogotovirus. The three influenza genera differ in their host range and pathogenicity. The primary natural reservoir host species of Influenza A virus (IAV) include shorebirds and wild aquatic waterfowl (Webster *et al.* 1992; Centers for Disease Control and Prevention (CDC) 2016), however IAVs have adapted to stably infect a wide variety of avian and mammalian species, whereas influenza B and C virus infection is primarily restricted to humans (Palese and Shaw 2007).

IAV is enveloped with a host cell-derived lipid membrane and contains eight different RNA gene segments, each encoding at least one protein. The envelope is covered with projections of three surface proteins: heamagglutinin (HA), neuraminidase (NA), and matrix 2 (M2). The internal proteins include the nucleoprotein (NP), the matrix protein (M1) and the polymerase complex, which is composed of the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA). Nonstructural protein NS1 and non-structural protein NS2, also known as nuclear export protein (NEP) are another set of proteins produced by IAV. NS1 has pleiotropic functions including dsRNA binding, inhibiting host mRNA processing, enhancement of viral mRNA translation

and type I interferon (IFN) antagonism (Palese and Shaw 2007; Gack *et al.* 2009). The protein PB1-F2 is not found in all IAV strains, but is thought to be involved in host cell apoptosis (Chen *et al.* 2001).

The antigenic characterization of the HA and NA surface glycoproteins allows for the subdivision of the different IAV strains into subtypes. There are currently 18 HA and 9 NA subtypes known, meaning theoretically there are 162 possible HA-NA combinations. The World Health Organization guidelines for the nomenclature of IAV include (1) antigenic type (A, B, or C); (2) host (if nonhuman) the virus was isolated from; (3) geographic region of the isolate; (4) unique isolation reference number; (5) year of isolation; and finally (6) the HA and NA subtypes. For example, A/Puerto Rico/8/1934 (H1N1) indicates type A, isolated in Puerto Rico with an isolate number 8 in 1934, with HA subtype 1 and NA subtype 1 (Taubenberger and Kash 2010), which is often abbreviated to PR8.

Viral life cycle

IAV is spread through aerosol exposure whereby it enters the respiratory tract. The first step of IAV infection is the attachment of the viral HA protein to sialic acid (SA) which are distal components of the glycosylation of host cell glycoproteins (Figure 1A). Sialic acids are a derivative of neuraminic acid and often represent terminal carbohydrate found in N- and O-linked gylycoproteins. Sialic acid molecules are classified by how they are linked to the underlying sugars by the α-2 carbon, with α-2,3 and α-2,6 being the most common linkages. Different IAVs have HAs with varying specificity to SAs. Avian upper airway epithelium expresses predominantly α-2,3 SA, whereas the human airway epithelium expresses mostly α-2,6 SA (Couceiro *et al.* 1993). Therefore, IAVs adapted to birds bind preferentially with α-

2,3 SA, and generally do not infect humans. However, some humans and avian hosts have been found to express both types of SAs, although with different tissue distributions (Thompson *et al.* 2006; Wan and Perez 2006). The α2,3 SA has been documented to be expressed on human alveolar cells from the lower respiratory tract, hence the potential for avian influenza infection of humans (Ibricevic *et al.* 2006; Shinya *et al.* 2006; Nicholls *et al.* 2007). Pathology of IAV can be attributed to this receptor distribution where highly pathogenic IAV strains such as H5N1 do not infect the upper respiratory tract where α-2,6 SA is abundant, but rather cause pneumonia by binding to α-2,3 SA in alveolar type II cells in the lower lung (Shinya *et al.* 2006).

Once the virus has attached to the host cell it is endocytosed by both clathrin-mediated and clathrin-independent mechanisms, and cleaved of HA by host proteases (Steinhauer 1999) (Figure 1B). Due to the requirement of these host proteases, the IAV tissue tropism is generally limited to the respiratory epithelial cells (Julkunen *et al.* 2000), although the infection of macrophages and leucocytes has also been documented this does not result in progeny (Manicassamy *et al.* 2010). In addition, some highly pathogenic IAV strains have polybasic HA cleavage sites, allowing for HA cleavage by a wider range of host proteases and thus IAV infection of cells beyond airway epithelium. The acidic pH in the late endosome triggers a conformational change in HA resulting in the fusion of the viral and endosomal membranes allowing for the release of the viral RNA into the cytoplasm (Steinhauer 1999) (Figure 1C, D). The M2 protein plays a critical role in this triggering process as it acts as a proton channel allowing H^+ ions to enter into the virion inducing the conformational change (Pinto and Lamb 2007) (Figure 1C).

Following uncoating, the eight RNA segments closely associated with the viral NP and the viral polymerase in viral ribonucleoproteins (vRNPs) are transported to the nucleus where the RNA undergoes genome replication and transcription (O'Neill *et al.* 1995; Bouvier and Palese 2008). The viral RNA polymerase, in combination with components of the host's transcriptional machinery, transcribes viral RNA (vRNA) into mRNA. The viral genome is replicated via positive strand complementary RNA (cRNA) synthesis within the nucleus (Figure 1E). The mRNA migrates to the cytoplasm to begin viral protein translation. The new strands of RNA are also transported to the cytoplasm for the formation of the new RNP, where each new strand of RNA is encapsulated by the viral nucleoprotein (Baudin *et al.* 1994). The viral polymerases and NP catalyse the genome replication and transcription (Neumann *et al.* 1999). The proteins M1 and NEP also play a crucial role in the trafficking of viral proteins to and from the nucleus. The newly produced virus components are then moved to the apical plasma membrane of the host where they are assembled and the process of budding begins (Figure 1G). The M1 protein is required for the assembly and structure of the virion (Bourmakina and García-Sastre 2003) (Figure 1F). For efficient budding to occur the NA protein needs to remove the sialic acid from the surface glycoproteins, specifically the HA protein. This prevents self-binding of the protein and aggregation of the virus (Matrosovich *et al.* 2004) (Figure 1H).

HA and NA are the antigenic targets of the humoral immune response to IAV, and the antiviral drugs oseltamivir and zanamivir target NA (Figure 1I). These viral sialidase inhibitors prevent the release of progeny virions and thereby limit the spread of infection. The adamantine class of antiviral drugs target the M2 protein to prevent viral replication (Taubenberger and Kash 2010) (Figure 1J). Ribavirin has

also been demonstrated to prevent viral replication by acting on the RNA polymerase function (Figure 1K).

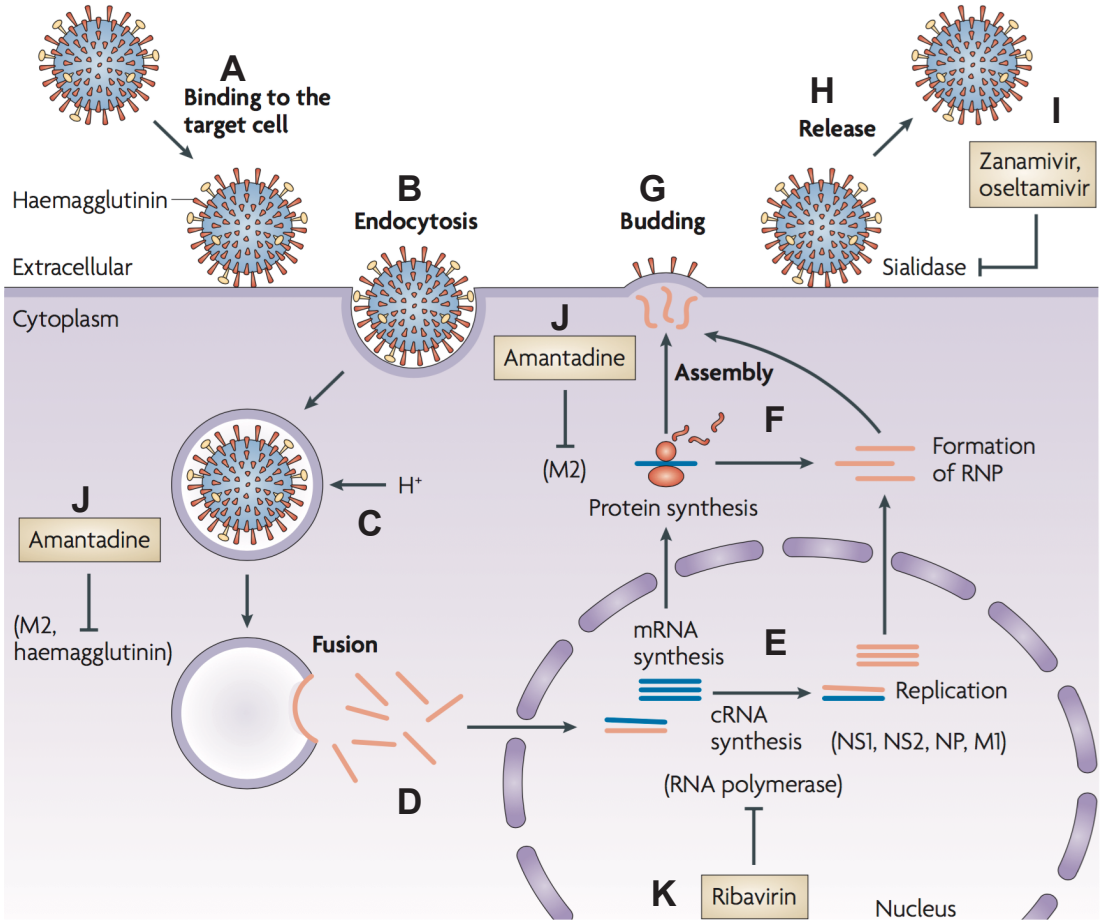


Figure 1 Life cycle of the influenza virus and targets for therapeutic intervention

IAV adheres to the target host cell using the viral surface glycoprotein haemagglutinin, which recognise host glycoconjugate residues (A). The virus is then endocytosed (B). A change in pH from the transport of ions into the vacuole containing the virus (C) allows for fusion and the release of viral RNA into the cytosol (D). The RNA is transported to the nucleus where viral RNA polymerase and the host-cell machinery are engaged to produce the necessary viral components (D). Viral genome is replicated via cRNA synthesis within the nucleus (E). The new viral proteins and RNA come together to form the new RNP where they are transported for assembly at the host cell plasma membrane (F). The budding process allows for new virion progeny to exit the host cell (G). Viral sialidase (NA) cleaves the host sialic acids to completely detach from the host cell to seek new host cells to infect (H). Zanamivir and oseltamivir efficiently block the sialidase activity of NA and the release of the virus from the host (I). Adamantanes target the M2 ion channel of the virus to prevent viral replication (J). Last, Ribavirin also prevents viral replication by targeting RNA polymerase.
Adapted from (Itzstein 2007). Permission to reproduce this figure has been granted by Nature publishing group.

Antigenic drift and Antigenic shift.

Influenza viruses have a high mutation rate and have the ability to reassort their gene segments, thus potentiating the large diversity in the viral population (Manrubia *et al.* 2005). IAV lacks a proofreading mechanism during the replication of viral RNA, which results in errors in transcription leading to this high mutation rate, known as antigenic drift (Palese and Shaw 2007). Many of these mutations however are deleterious to the virus as it can create premature stop codons, changes in the regulator signal that affects replication, or changes to the amino acids which render the virus less fit (Pybus *et al.* 2007). Amino acid mutations in the antigenic epitopes of the HA and NA may allow the IAV to evade pre-existing or newly forming immunity. Mutations of the HA also makes vaccination difficult as vaccine protection is mediated primarily by specific antibodies being produced to the specific HA subtype.

Co-infections of one host cell with two or more different IAVs can result in progeny viruses containing segments from the different parental viruses. This generally occurs within pigs, which are known as the mixing vessel for IAV. When this re-assortment results in different HA or NA subtypes this is classified as antigenic shift (Taubenberger and Kash 2010). When a human host becomes exposed to a new HA or NA subtype there is potentially little to no protective immunity and the virus can spread rapidly, causing a widespread and potentially severe pandemic outbreak.

Epidemics and Pandemics

The ability of the virus to mutate and reassert its genetic segments allows for the outbreak of epidemics and pandemics. Seasonal epidemics are estimated by the

World Health Organisation (WHO) to cause three to five million cases of severe illness and about 250,000 to 500,000 deaths annually worldwide (WHO 2014). The current circulating strains are generally restricted to the IAV strains H1N1 and H3N2 (Kreijtz *et al.* 2011).

Pandemic viruses have been isolated from 1957 onwards, with the complete reconstruction of the 1918 pandemic virus occurring in 2005 (Taubenberger *et al.* 2005). There have been five notable pandemics in the past century: 1918 H1N1 'Spanish flu', 1957 H2N2 'Asian flu', 1968 H3N2 'Hong Kong flu', and the most recent 2009 H1N1 'swine flu'. The 1918-1919 'Spanish flu' was the most severe documented pandemic when a novel avian-descended H1N1 virus emerged, resulting in an estimated 50 million deaths (Johnson and Mueller 2002; Taubenberger *et al.* 2005; Rabadan *et al.* 2006). A novel H1N1 virus derived from two distinct swine H1N1 viruses caused the recent 2009 'swine flu' pandemic (Garten *et al.* 2009), resulting in approximately 148,000 – 249,000 deaths worldwide (Simonsen *et al.* 2013).

IAV Host Switch

The mechanisms by which avian IAVs cross the species barrier to infect mammals are not fully known. The growing number of human zoonotic infections has been associated with a high mortality rate leading to great concern of a possible future pandemic. Two subtypes of the HA (H5 and H7) are known to give rise to the highly pathogenic avian influenza (HPAI) viruses in poultry (Peiris *et al.* 2007). Since 1997, there have been a total of 720 reported cases of HPAI H5N1 infections in humans, predominantly in Southeast Asia (Peiris *et al.* 2007; Uyeki and Bresee 2007; Qin *et al.* 2015), of which 60% proved to be fatal (Kreijtz *et al.* 2011). 55 family clusters

were found among the reported human HPAI H5N1 cases reported (Qin *et al.* 2015). This highlights the possibility of human-to-human H5N1 transmission. In fact, household secondary attack rates (SAR) were estimated at 29% (Uyeki and Bresee 2007), which is similar to the SAR estimates from IAV infections in the United States (12.7-30.6%) (Longini *et al.* 1988). Although overshadowed by the spread of H5N1, H5, H7, and H9 subtype HPAI viruses, which are unrelated to the Asian H5N1, have also been isolated from infected humans (Alexander 2007; de Wit *et al.* 2008).

1.1.2 Streptococcus pneumoniae

Streptococcus pneumoniae is a gram-positive extracellular bacterium that transiently colonises the mucosal surfaces of the upper respiratory tract. It is the most common bacterial respiratory pathogen, frequently causing invasive disease leading to meningitis, otitis media, sepsis and pneumonia in the young, old and immunocompromised. *S. pneumoniae*-induced disease is the major cause of infant mortality, with a mortality rate of approximately 20-25% in children under the age of 5 in the developing world and 10% worldwide, resulting in more than 1.2 million deaths annually worldwide (Berkley *et al.* 2005). Depending on the age of the host, 30-60% of survivors develop long term clinical sequelae, such as neurological deficits, hearing loss and neuropsychological impairment (Mook-Kanamori *et al.* 2011). There are currently 91 known pneumococcal capsular serotypes of *S. pneumoniae*, which vary in their degree of carriage and virulence (Kadioglu *et al.* 2008). This variation of serotypes makes vaccination difficult. The 23-valent

vaccine, which has a theoretical coverage of 85-90% of the circulating strains, is only immunogenic in adults and children over the age of 5. The polysaccharides contained within this vaccine induce a severely impaired antibody response in children younger than 2 years old (Koskela *et al.* 1986; O'Brien *et al.* 1996). Fortunately, recent conjugate vaccines are highly immunogenic in children younger than 2 years of age, even inducing immunological memory (Eskola and Anttila 1999). Penicillin is typically used to treat *Streptococcus pneumoniae* infections, however antibiotic resistance is increasing (Henriques-Normark and Tuomanen 2013).

Mechanism of Colonisation

S. pneumoniae resides asymptomatically in the human nasopharynx, however spread of the bacteria to normally sterile areas of the airway can lead to severe complications and disease (Kadioglu *et al.* 2008). Colonisation generally occurs within early childhood. Once inhaled, *S. pneumoniae* enters the nasal cavity and encounters the mucus secretions. A polysaccharide capsule covers the outer surface of the pneumococcus. This capsule reduces the entrapment within the mucus and prevents phagocytosis of the bacterium, thereby facilitating epithelial attachment (Nelson *et al.* 2006). A thick capsule however, inhibits the ability to adhere to the epithelial surface. Most pneumococcal strains therefore display a phase variation of the capsular gene. The two bacterial forms can be distinguished by their opaque or transparent colony morphologies (Weiser *et al.* 1994). The opaque phenotype is associated with more capsular polysaccharide than the transparent form. During initial colonisation, transparent variants display increased binding to host tissues in comparison to the opaque variants (Weiser *et al.* 1994).

The adherence to the epithelial lining of the respiratory tract is essential for colonisation. Asymptomatic colonisation requires binding to cell-surface carbohydrates (N-acetyl-glycosamine) on resting, non-inflamed epithelia (Bogaert *et al.* 2005). The bacterial surface molecules that bind to these host sugars include: lipoteichoic acids, pneumococcal surface adhesion A (PsaA), choline-binding protein A (CbpA) and phosphorylcholine (ChoP) (Bogaert *et al.* 2005; Kadioglu *et al.* 2008). ChoP mediates bacterial adherence by binding to the platelet activating-factor receptor (PAFR). CbpA shows an increased affinity for immobilised sialic acid and lacto-N-neotetraose. Binding of these bacterial components to these host receptors induces internalisation of pneumococci and promotes transepithelial migrations, resulting in bacterial invasion of the organism (Bogaert *et al.* 2005). *S. pneumoniae* can also produce three exoglycosidases: a neuraminidase, NanA, a β-*N*-acetylglucosaminidase, StrH, and a β-galactosidase, BgaA (King *et al.* 2009). These exoglycosidases may enhance colonisation due to the cleavage of the N-acetylneuraminic acid, which is the most predominant sialic acid found in mammalian mucus. This cleavage reduces the viscosity of the mucus, allowing for the bacteria to reach the epithelial surface. These exoglycosidases can also remove the terminal sugars found on many human glucoconjugates, which may allow for the exposure of adherence receptors (Tong *et al.* 2000; Kadioglu *et al.* 2008). Overall, the bacteria employ all of these mechanisms to penetrate through the mucus and bind to the airway epithelial cells to colonize a host.

1.1.3 IAV-*S. pneumoniae* co-infection

There is increasing clinical evidence that a viral infection followed by a bacterial infection results in exacerbated disease, leading to increased morbidity and mortality compared to either single pathogen (Madhi *et al.* 2004; Thorburn *et al.* 2006). The IAV pandemics since 1918 have demonstrated this association convincingly. During the 1918 'Spanish flu', 95% of fatal cases were attributed to a bacterial co-infection (Morens *et al.* 2008). Of the bacteria isolated from the lungs of those that succumbed to disease, *Streptococcus pneumoniae* was found to be the most prevalent (Morens *et al.* 2008). *Staphylococcus aureus, Haemophilus influenzae and Streptococcus pyogenes* were also all attributed to be the most predominant bacteria in separate studies (Brundage and Shanks 2008). Of the 1957 and 1968 pandemics, where the death toll was less severe compared to the 1918 pandemic, co-infection was attributed to only 20-44% of deaths (Collins and Lehmann 1951; Dauer 1958). *Staphylococcus aureus* was the most prevalent bacterium in the bacterial isolates from these individuals, who were mainly patients with chronic medical conditions. This may reflect the use of antibiotics which are effective against *S. pneumoniae*, and the emergence of drug-resistant bacteria (Robertson *et al.* 1958; McCullers 2006). A similar trend was observed during the 2009 pandemic. In the United States, 13% of the bacteria isolated from *post mortem* lung samples were found to be *S. pneumoniae* (Louie *et al.* 2009), and in Japan and Argentina, this percentage increased to approximately 50% (Palacios *et al.* 2009; Okada *et al.* 2011). This trend is not only observed in pandemics but also with seasonal outbreaks of IAV. For example, American children hospitalised with pneumococcal pneumonia were significantly more likely than control children to

have displayed IAV-induced symptoms in the weeks prior to their hospitalisation (O'Brien *et al.* 2000).

It is therefore of great importance to understand the mechanisms behind the induction of exacerbated disease and death during co-infection, and their identification will allow for generation of better treatments against co-infections. Animal mouse studies have identified a number of these mechanisms, which will be discussed further within this Chapter and in Chapter 3. However, the initial and main inducers of many of these effects remain to be identified, and airway epithelial cells represent prime candidates for this.

1.2 The Immune response to IAV

1.2.1 Innate immune response to IAV

Recognition of IAV

The innate immune system is the first line of immune defence against invading pathogens. IAV is recognised through pathogen associated molecular patterns (PAMPs), which are present within the virus, by pattern recognition receptors (PRRs). Recognition of the invading virus induces a rapid, broadly reactive response to reduce and control the infection. IAV is recognised by at least 3 distinct classes of PRRs: Toll-like receptors (TLR), NOD-like receptors (NLR) and retinoic acid-inducible gene I (RIG-I).

There are 11 TLRs, with some expressed on the cell surface (TLR1, 2, 4, 5, 6, 10 and 11) and the others on the luminal side of endosomes (TLR3, 7, 8 and 9), which bind to a variety of viral and bacterial PAMPS (Schmolke and García-Sastre 2010). Specifically for detection of IAV, TLR3, which recognises double-stranded RNA (dsRNA) (Guillot *et al.* 2005), TLR7 and TLR8, which recognise single-stranded RNA, are utilised by host cells.

IAV infected cells do not generate dsRNA (Pichlmair *et al.* 2006), therefore it is thought that activation of TLR3 occurs due to currently unidentified RNA structures produced during phagocytosis of dying IAV-infected cells (Schulz *et al.* 2005). TLR3 is constitutively expressed in human epithelial cells and, once activated by IAV, induces the activation of the transcription factors IFN regulator factor 3 (IRF3) and nuclear factor-κB (NFκB) (Kawai and Akira 2007). Activation of these pathways leads to the production of type I and III IFNs and the production of proinflammatory cytokines, respectively (Le Goffic *et al.* 2007).

Plasmacytoid dendritic cells (pDCs) utilise TLR7 to recognise ssRNA genomes contained within IAV once they enter the endosome (Diebold 2004; Lund *et al.* 2004). Like TLR3, signalling through this receptor induces the activation of NFκB and IFN-regulator factor 7 (IRF7), which again are responsible for the expression of proinflammatory cytokines and type I and III IFNs. TLR7 was also implicated in eliciting a robust antibody response to IAV (Heer *et al.* 2007). TLR8, which is expressed by human monocytes and macrophages, can induce the proinflammatory cytokine IL-12 but not IFNα, a type I IFN, following activation (Ablasser *et al.* 2009). However, the exact relevance of this receptor during IAV infection remains to be elucidated.

Activation of the NLR pathways induces formation of inflammasome complexes resulting in the autocatalytic activation of pro-caspase 1, which can elicit pyroptosis of infected cells (Bergsbaken *et al.* 2009) and generate mature IL-1β and IL-18. The NLRP3 system can be activated by numerous stimuli including a variety of pathogens, stress and host cell damage (Martinon *et al.* 2009). The involvement of the inflammasome signalling after IAV infection in the innate immune response has been demonstrated (Allen *et al.* 2009; Thomas *et al.* 2009). However, the study by Allen *et al*. inoculated mice with IAV along with alum, which is well known for inducing the NLRP3 inflammasome (Li *et al.* 2008). Therefore the study by Allen and colleagues does not fully demonstrate that it is IAV alone that is inducing the inflammasome signalling. Monocytes, DCs, neutrophils, macrophages (Guarda *et al.* 2011), and bronchial epithelial cells (Pothlichet *et al.* 2013) have all been shown to undergo NLRP3 signalling. One study showed the inflammasome recognition of IAV is essential for the adaptive immune response to infection (Ichinohe *et al.* 2009).

RIG-I activation is crucial for IAV detection, and type I and III IFN production in infected epithelial cells, conventional dendritic cells (cDCs) and alveolar macrophages (Kato *et al.* 2005). RIG-I recognises the 5'-triphosphorylated viral ssRNA, the 5' pattern of viral genomic RNA, within the cytosol of infected cells (Hornung *et al.* 2006; Pichlmair *et al.* 2006; Baum *et al.* 2010; Rehwinkel *et al.* 2010). Upon recognition of IAV, a conformation change enables the caspase-recruitment domains to bind to the signalling adaptor: mitochondrial antiviral signalling protein (MAVS) (Jiang *et al.* 2011; Kowalinski *et al.* 2011). MAVS subsequently activates IRF3, IRF7 and NFκB, initiating the IFN and inflammatory response to infection. NS1 protein can bind to viral RNA, masking it from PRRs recognition. NS1 proteins can also block RIG-I binding to the tripartite-motif-containing protein 25 (TRIM25), therefore suppressing RIG-I signalling and IFN production (Gack *et al.* 2009). The evolution of IAV to antagonise RIG-I and IFN signalling by NS1 demonstrates the importance of the RIG-I mediated recognition of IAV and the IFN antiviral response to IAV (Pichlmair *et al.* 2006; Palese and Shaw 2007; Gack *et al.* 2009).

IFNαβ and IFNλ response

IFNs were discovered and named for their ability to 'interfere' with viral replication. There are three distinct families of IFNs: type I, type II and type III. The type I IFN family is a multi-gene cytokine family consisting of 13 partially homologous IFNα subtypes (11 in mice), a single IFNβ, and several other family members (IFNε, IFNδ, IFNκ, IFNω, and IFNζ). The type II IFN family contains IFNγ. The final family of type III IFNs comprises of IFNλ1 (IL-29), IFNλ2 (IL28A), IFNλ3 (IL28B) and the

recently discovered IFNλ4 (Pestka *et al.* 2004; Prokunina-Olsson *et al.* 2013; O'Brien *et al.* 2014).

As mentioned, type I IFNs are induced after IAV detection by PRRs. Almost all cells in the body have the capacity to produce and respond to type I IFNs. pDCs were found to be the most potent type I IFN producer (Ganguly *et al.* 2013). Type I IFNs are classified as antiviral cytokines, capable of inhibiting replication and spread of viruses (Isaacs and Lindenmann 1957; Müller *et al.* 1994). All type I IFNs signal exclusively through the ubiquitously expressed heterodimeric transmembrane receptor, IFNαβR. This receptor is composed of the subunits IFNAR1 and IFNAR2 (McNab *et al.* 2015). IFNα and IFNβ are more broadly expressed than the other family members, and therefore many studies concentrate on the actions of these two subtypes. IFNαβ can act in an autocrine and paracrine manner by binding to the IFNαβR on the infected cell or to neighboring cells (Randall and Goodbourn 2008). Engagement of the receptor activates janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). JAK1 and TYK2 then act to phosphorylate signal transducers and activators of the transcription 1 and 2 (STAT1 and STAT2) present in the cytosol, leading to dimerization and binding to IRF9 to form the trimeric transcription factor ISGF3 (Murray 2007). The ISGF3 complex binds to IFN-stimulated response elements which triggers the expression of several hundred interferon stimulated genes (ISGs) (Ivashkiv and Donlin 2014; Schneider *et al.* 2014)

Many of these ISGs have been attributed with a direct antiviral role, by inhibiting viral replication and limiting viral spread, including the MX, IFITM, OAS and RSAD protein families. MX proteins were amongst the first ISGs to be identified that restrict IAV infection (Staeheli *et al.* 1986). Humans express MXA

and MXB, however it is only the MXA protein, which is present in the cytosol, that has potent antiviral activity towards IAV, along with many other viruses (Pavlovic *et al.* 1995; Hefti *et al.* 1999; Turan 2004). In contrast, the MXB protein, present at the cytoplasmic face of nuclear pore complexes, does not inhibit IAV infection but can restrict human immunodeficiency virus-1 (HIV-1) (Liu, Pan, *et al.* 2013). IFITM proteins, which are constitutively expressed by respiratory epithelial cells, macrophages, and endothelial cells, also restrict IAV replication by blocking virus to host cell membrane fusion (Huang *et al.* 2011). Furthermore, IFITM3 was found to have a role in limiting host damage resulting from IAV infection (Everitt *et al.* 2012). Humans can have natural hypomorphic mutations in the *ifitm3* gene which associates with increased morbidity and mortality following IAV infections (Everitt *et al.* 2012). The OAS (2'-5'-oligoadenylate synthase) family acts together with ribonuclease L (RNase L) to degrade viral RNA present in the cytosol (Iwasaki and Pillai 2014). Finally, overexpression of RSAD2 (viperin) blocks the release of IAV from host cells by inhibiting the formation of lipid rafts (Wang *et al.* 2007).

Type I IFNs not only induce a cell-intrinsic antiviral response but are also involved in activating or inhibiting the response of myeloid cells, NK cells, B cells and T cells, depending on the response context. Type I IFN production can activate immature committed dendritic cells (DCs), enhancing the cell surface expression of major histocompatibility complex (MHC) along with the co-stimulatory molecules CD80 and CD86, to increase the ability to stimulate T cells and thereby viral clearance (Montoya *et al.* 2002). It has also been observed that type I IFN can promote the DC capability to cross-present antigens and migrate to the lymph nodes during viral infections (Parlato *et al.* 2001; Le Bon *et al.* 2003). NK cells were found to have increased function and survival following stimulation with type I IFNs.

Following IAV infection, the type I IFNs produced induce the cytolytic effector function and the production of IFNγ by NK cells (Nguyen *et al.* 2002). B cell activation and antibody responses were increased following type I IFN stimulation early in IAV infection, however late in infection the role of type I IFNs on B cells is negligible (Price *et al.* 2000; Swanson *et al.* 2010). A stimulatory and inhibitory role of type I IFNs have also been described for both $CD4^+$ and $CD8^+$ T cell survival, proliferation, cytokine production, cytotoxic function and memory formation (McNab *et al.* 2015).

Although type I IFNs are associated with a protective antiviral response, the immunomodulatory effects, along with triggering inflammation and tissue damage, may sometimes supersede their protective actions resulting in exacerbated disease. IAV mediated lung tissue damage may be due to the viral cytotoxic effects, or to the immune response. If the immune response is left unchecked it can potentially cause pathology to the host. This was observed in patients infected with highly pathogenic influenza strains, where high proinflammatory cytokines associated with tissue damage and disease severity (Peiris *et al.* 2004; de Jong *et al.* 2006). Two studies have delved specifically into the role of type I IFNs during influenza infection with both associating high type I IFN concentrations with increased disease severity (Boon *et al.* 2009; Davidson *et al.* 2014). Type I IFNs have been demonstrated to regulate not only cellular survival but also have the potential to induce cell death pathways (Leaman *et al.* 2003). The apoptosis inducing ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is upregulated on many cells following type I IFN stimulation during IAV infection (Herold *et al.* 2008). This member of the TNF superfamily (TNFSF10) of cytokines can act as a surface-bound or secreted protein that upon binding with its receptors (TRAIL-R1 and

TRAIL-R2 in humans, and DR5 in mice) induces apoptosis of the cells in a caspase-dependent manner (Walczak *et al.* 1997; Holoch and Griffith 2009; Benedict and Ware 2012). In IAV infection, type I IFN mediated upregulation of TRAIL on inflammatory monocytes, and DR5 on airway epithelial cells resulted in epithelial cell death and lung damage leading to host morbidity and mortality (Herold *et al.* 2008; Davidson *et al.* 2014). FASL, another apoptosis inducing ligand, which can bind to its receptor FAS to induce apoptosis, was found to be induced following type I IFN stimulation during IAV infection, and also correlated with lung damage and disease severity (Fujikura *et al.* 2013). Overall, the immunomodulatory effects of type I IFNs on immune cells which act in parallel to the induction of ISGs, shows the potential of type I IFNs to over-activate the immune system during IAV infection, inducing immunopathology. However, induction of ISGs with the potential to be pathogenic, such as TRAIL, requires 100-fold higher IFN concentrations than that of antiviral ISGs such as MX (Leaman *et al.* 2003; Thomas *et al.* 2011; Davidson *et al.* 2015).

Type III IFNs were discovered in 2002 (Kotenko *et al.* 2002; Sheppard *et al.* 2002). Like type I IFNs, type III IFNs induce the same JAK/STAT signalling pathway to activate transcription of the same set of ISGs (Sheppard *et al.* 2002; Crotta *et al.* 2013). However, type III IFNs bind to a completely separate receptor, IFNλR, which is composed of IFNλR1 and IL-10 receptor chain 2 (IL-10R2). This receptor, unlike the IFNαβR, is restricted to mucosal surfaces such as the airway epithelium or the gut (Sommereyns *et al.* 2008; Mordstein *et al.* 2010). Due to this receptor distribution, type III IFNs cannot stimulate the immune cells of the host. Infection of mice deficient in IFNAR, IFNλR, or both receptors, with a variety of viruses has established redundancy between these two types of IFN. Only when

there was absence of both receptors was a dramatic loss of viral control and host mortality observed (Mordstein *et al.* 2008; Crotta *et al.* 2013). This is only true for viruses for which tissue tropism is restricted to epithelial cells such as IAV. IAV can only productively infect epithelial cells, whereas many other virus such as the measles virus can productively infect both epithelial and immune cells (Tahara *et al.* 2008). In this case loss of IFNAR may be detrimental. Type III IFNs may therefore have evolved to induce an antiviral state within the epithelial cells, the first host target of infection for many viruses, without triggering the immune system, which as previously discussed can induce immunopathology.

1.2.2 Overview of adaptive immune response to IAV

The innate immune response is crucial for the recognition and initial control of IAV. It is the adaptive immune response however that is essential to fully clear the infection. The lung antigen-presenting cells (APCs) such as DCs are recruited, activated, and uptake antigen following IAV detection. These cells then migrate out of the infected lungs to the draining lymph nodes (Holt *et al.* 2008). The accumulation of APCs in the draining lymph node concentrates viral antigens where virus-specific naïve T cells reside, thus promoting the encounter with their cognate antigens and their subsequent priming (Khanna *et al.* 2008). Virus specific $CD8^+$ T cells recognise infected cells via influenza peptide-MHC class I (MHCI) complexes on the cell surface (Thomas *et al.* 2006). Once IAV is detected, activated $CD8^+$ T cells lyse the infected cells through perforin, FAS-, or TRAIL-dependent mechanisms (Topham *et al.* 1997; Brincks *et al.* 2008). Human studies

have revealed that IAV-specific $CD8^+$ T cells react to the viral NP and M1 proteins (Gotch *et al.* 1987; Jameson *et al.* 1998). These proteins are highly conserved between IAV strains, therefore allowing for a higher degree of cross-protection by $CD8^+$ T cells between the different strains. Due to this selective pressure, many amino acid substitutions occurring during the evolution of the H3N2 strain associated with decreased recognition by virus-specific $CD8^+$ T cells (Boon *et al.* 2004; Berkhoff *et al.* 2007).

The primary role of $CD4^+$ T cells in IAV infection is to provide cognate help to B cells through TCR:MHCII interactions, promoting activation of B cells, class switching, and affinity maturation of the antibodies they produce. This T-cell help promotes the antibody response against IAV (Topham *et al.* 1996; Topham and Doherty 1998). Most protective antibodies induced by IAV infection however are mainly directed to the highly variable head region of HA and are therefore often strain specific (Caton *et al.* 1982; Laursen and Wilson 2013). These antibodies can mark the infected cell for destruction by phagocytic cells, known as antibody-dependent cell-mediated cytotoxicity (ADCC) (Hashimoto *et al.* 1983). HA specific antibodies can also neutralise the ability of IAV to attach to and infect the host cells (neutralising antibodies). The high variability of the HA head region is immune-driven and makes generation of effective vaccines difficult, as they cannot induce broadly neutralising antibodies. Several cross-reactive antibodies against IAV have been documented. HA-stalk reactive antibodies can bind to highly conserved epitopes in the membrane-proximal stalk region of the HA molecule to neutralise many different HA subtypes (Ekiert *et al.* 2009; 2011; Tan *et al.* 2012; DiLillo *et al.* 2014; Tan *et al.* 2014). These broadly neutralising antibodies work either by preventing fusion with the host cell (Ekiert *et al.* 2009), by preventing IAV from

escaping from within the endosome (Tan *et al.* 2012), or by hindering virus budding (Tan *et al.* 2014). Additionally, antibodies to NA, M2, and NP have been shown to also have broad neutralising or ADCC-inducing capacity against IAV (Zebedee and Lamb 1988; Mozdzanowska *et al.* 1999; Carragher *et al.* 2008).

$CD4^+$ T cells, $CD8^+$ T cells, antibodies, and memory B cells are all therefore essential for clearance and immunological memory against IAV. As previously discussed, antigenic drift can allow for IAV to evade the adaptive responses due to reduced affinity of the antibodies and $CD8^+$ T cells. Antigenic shift is another mechanism by which IAV evades the adaptive immune response, as the host may have no pre-existing antibodies or T cells able to recognise the completely novel IAV strain.

1.3 The Immune response to *S. pneumoniae*

1.3.1 Overview of innate immune response to *S. pneumoniae*

Like IAV, PAMPS of *S. pneumoniae* are detected by the PRRs of the host, including TLRs, NLRs, and C-type lectins. TLR2 recognises the cell wall peptidoglycan and lipoteichoic acid (LTA) of *S. pneumoniae* (Yoshimura *et al.* 1999; Han *et al.* 2003; Schroder *et al.* 2003). However, studies on the role of TLR2 in pneumococcal pneumonia have shown only a modest contribution of this receptor in the defence against the pathogen (Knapp *et al.* 2004). In contrast, TLR2 was found to play a detrimental role during infection by impairing the epithelial barrier integrity and promoting pneumococcal translocation (Clarke *et al.* 2011). TLR4, a receptor that is traditionally associated with the recognition of gram-negative bacteria, can directly interact with the pneumolysin of *S. pneumoniae* (Srivastava *et al.* 2005). The importance of TLR4 in the detection of the bacterium appears to be restricted to the airway surfaces, as one study found no difference in blood bacterial loads, and survival in TLR4 deficient mice following infection (Benton *et al.* 1997). TLR9 detects unmethylated cytosine-phosphate-guanosine (CpG) motifs which are abundant in prokaryotic DNA (Bauer *et al.* 2001). Studies have shown only a mild increase in disease severity when any one of these three TLRs were missing (Knapp *et al.* 2004; van Rossum *et al.* 2005), but mice lacking the adaptor molecule myeloid differentiation factor 88 (MyD88) were highly susceptible to infection (Albiger *et al.* 2005). MyD88 is a key adaptor molecule in the signalling cascade downstream of nearly all TLRs, except TLR3 (Premkumar *et al.* 2010), and of the interleukin-1 receptor (IL-1R) and IL-18R. TLRs therefore play an important but a partially redundant role in the recognition of *S. pneumoniae*.

Nod1 and Nod2 recognize microbial products to activate a NFκB dependent proinflammatory cytokine response (Chen *et al.* 2009). Nod1 detects peptidoglycan fragments which are mainly produced by gram-negative bacteria, whereas Nod2 detects peptidoglycans of most bacteria, including *S. pneumoniae* (Opitz *et al.* 2004). This recognition of *S. pneumoniae* by Nod2 requires phagocytosis and lysozyme-dependent digestion of the bacteria by macrophages. The subsequent delivery of pneumococcal peptidoglycan fragments into the cytosol of the host induces Nod2 activation (Opitz *et al.* 2004; Davis *et al.* 2011). NLRP3 has also been attributed with the response to *S. pneumoniae.* The NLRP3 pathway was shown to be involved in the pneumolysin mediated proinflammatory cytokine production in macrophages and DCs (McNeela *et al.* 2010; Witzenrath *et al.* 2011).

The C-type lectin SIGN-R1 is expressed by macrophages and can bind to capsular polysaccharide of the bacteria (Kang *et al.* 2004). The absence of SIGN-R1 results in sepsis and increased mortality following *S. pneumoniae* infection of mice (Lanoue *et al.* 2004; Koppel *et al.* 2005). Another macrophage receptor, MARCO can also bind to the bacteria to facilitate phagocytosis, although the bacterial ligands recognised by MARCO have yet to be identified (Arredouani *et al.* 2004).

Activation of these PRRs induces the expression of inflammatory mediators including TNF-α, IL-6, CXCL1, CXCL2, CCL2, IL-1β, and IFNαβ through the stimulation of NFκB and IRF3/7. These cytokines then activate and recruit immune cells such as neutrophils and macrophages, which phagocytose or kill the bacteria in an effort to clear the infection.

Like in IAV, type I IFNs can have both a protective and a detrimental role within bacterial infections. In studies utilizing the *Ifnar-/-* mice, infection with *S.*

pneumoniae resulted in increased bacterial growth and mortality compared to the wild-type controls (Weigent *et al.* 1986; Parker *et al.* 2014). Type I IFN signalling during *S. pneumoniae* infection contributes to the optimal secretion of TNF-α and inducible nitric oxide synthase (iNOS) from macrophages, and other antimicrobial effectors such as indoleamine 2,3-dioxygenase (IDO), granulocyte-binding proteins and proinflammatory cytokines (McNab *et al.* 2015). In contrast, type I IFNs may impair the host response to the bacteria by inducing extensive cell apoptosis such as TRAIL-DR5 interaction (Halaas *et al.* 2004), inducing IL-10 and IL-1 receptor antagonist to suppress the proinflammatory cytokine production, and reducing the responsiveness of immune cells to IFNγ (McNab *et al.* 2015).

The complement pathway is thought to be one of the most important immune response for the clearance of *S. pneumoniae*. C-reactive protein (CRP) binds to ChoP on the bacterium, which then binds to C1q of the complement system, activating the classical pathway of complement (Brown *et al.* 2002). Furthermore, the CRP concentrations found in the airway can inhibit the pneumococcal adherence to PAFR on the epithelial surface (Gould and Weiser 2002). The subsequent deposition and activation of the complement component, C3, on the surface of the bacteria results in the complement cascade leading to opsonphagocytosis of the bacterium and the induction of inflammation. As the complement system is critical in the immune response to the bacterium, pneumococcus has evolved several mechanisms to evade this response. The capsule limits the accessibility for the cell-bound complement and reduces the amount that can be deposited (Abeyta *et al.* 2003). PhpA on the surface of *S. pneumoniae* can degrade C3, thus preventing the complement-mediated clearance of the bacteria (Angel *et al.* 1994; Zhang *et al.* 2001). Two more surface proteins:

PspA and PspC, can also contribute to resistance from complement (Ren *et al.* 2004). Furthermore, pneumolysin has been found to protect from complement deposition and complement-mediated clearance (Yuste *et al.* 2005), however the mechanisms for how this protective response is initiated is unknown.

1.3.2 Overview of adaptive response to *S. pneumoniae*

The complement pathway can also be initiated by antibody binding. Antibody mediated complement-dependent opsonophagocytosis is thought to be the main method of *S. pneumoniae* control (Mold *et al.* 2002). The presence of serotype-specific IgG against the capsule of *S. pneumoniae* correlates with protection, which is the immune status that the 23-valent vaccine attempts to generate. Antibodies to the capsular polysaccharide of *S. pneumoniae* can agglutinate the bacteria and mark them up for phagocytosis (Musher *et al.* 1993). Preformed 'natural antibodies', which are produced without prior *S. pneumoniae* infection, but in response to the normal gut flora, can be protective against pneumococcal challenge (Mold *et al.* 2002). Furthermore, $CD4^+$ T cells have been found to be recruited rapidly to infected areas of the host in a pneumolysin-dependent manner, and contribute to bacterial clearance through induction of mucosal antibodies and a Th1 response (Kemp *et al.* 2002; van Rossum *et al.* 2005; Zhang *et al.* 2006).

1.4 Immune response to IAV-*S. pneumoniae* co-infection

IAV infection followed by bacterial infection, such as with *S. pneumoniae*, results in exacerbated disease leading to a more severe outcome in comparison to either single infection. Mechanistic studies of IAV-*S. pneumoniae* co-infections have implicated the primary viral infection predisposing the host to the secondary bacterial infection. Lung damage and dysfunction, increased epithelial adhesion, immune impairment and a strong proinflammatory cytokine response have all been attributed to the synergistic effects of co-infection. Mouse models of co-infection have also shown that the susceptibility to secondary infection occurs between 3 to 14 days post IAV infection, with the greatest susceptibility occurring at day 7 (McCullers and Rehg 2002).

1.4.1 Lung damage and dysfunction

Epithelial cell damage and reduced function

The primary IAV infection can cause many changes within the lung, which can facilitate the secondary bacterial invasion. IAV strains that express the pro-apoptotic viral peptide PB1-F2 induce lung damage and inflammation and associate with a more severe outcome following a co-infection (McAuley *et al.* 2007). This outcome depends on the sequence of PB1-F2, therefore disease severity correlates with the variant PB1-F2 present in the IAV strain. (Weeks-Gorospe *et al.* 2012; Alymova *et al.* 2013).

IAV infection creates epithelial damage leading to the sloughing of host cells into the airways. This along with increased mucus production provides an

environment rich in nutrients ideal for rapid bacterial growth (Siegel *et al.* 2014). The ciliated cells of the lung can also be affected by IAV infection, resulting in decreased ciliar beat frequency and uncoordinated movement (Levandowski *et al.* 1985; Pittet *et al.* 2010). This may reduce the mechanical clearance of the secondary pneumococcus, thus further potentiating *S. pneumoniae* colonization within the lung (Figure 2A).

Increased epithelial adhesion

Virus-mediated cytotoxicity or immune-mediated epithelial cell apoptosis leads to damage to the epithelium, exposing the extracellular matrix molecules and the basement membrane elements which bacteria can adhere to (McCullers 2004). This may allow for increased pneumococcal binding to the lung epithelium. Primary IAV infection was shown to increase the capability of *S. pneumoniae* to bind to mouse tracheal epithelial cells (Plotkowski *et al.* 1986) and a human alveolar cell line (McCullers 2004). Within the lungs of humans that succumbed to pandemic IAV, increased bacterial adherence was also observed (Morens *et al.* 2008; Centers for Disease Control and Prevention (CDC) 2009).

Most seasonal strains of IAV do not cause extensive lung damage but can still facilitate bacterial co-infections, although not as severe in comparison to the induction by high pathogenic IAV strains (Guarner and Falcón-Escobedo 2009). This suggests that other, potentially receptor-mediated, mechanisms are at play beyond the apoptosis-inducing mechanisms of high pathogenic IAV strains. The neuraminidase of the virus can cleave host SAs, which may expose the bacterial adhesion receptors. Furthermore, the viral neuraminidase can disrupt the sialylated mucins of the host that may have otherwise acted as decoy receptors for the

bacteria (McCullers and Bartmess 2003). *S. pneumoniae* also processes a neuraminidase to cleave the protective mucins and access the epithelial surface. The presences of IAV in the lungs before the inhaled bacteria may therefore generate an environment permissive for colonisation and unchecked outgrowth of the bacteria. The epithelial receptor PAFR may be one of these bacterial binding sites exposed following viral infection, allowing for binding by the virulence factor ChoP of *S. pneumoniae*. *Pafr-/-* mice had a moderately reduced bacterial load and were slightly protected (van der Sluijs *et al.* 2010).

Not only may there be increased lung damage and neuraminidase-mediated exposure of binding partners, but the repair and regeneration of the epithelial cell layer may also be affected. Kash and colleagues found that following co-infection there was a reduction in epithelial stem cells, proliferation, and reduction of the epithelial growth factors: hepatocyte growth factor (HGF) and fibroblast growth factor (FGF). The loss of these factors results in decreased repair responses (Kash *et al.* 2011). This may also lead to increased binding by *S. pneumoniae* as areas of incomplete repair have the basement membrane of the epithelia exposed, leaving components such as laminin or type I and type IV collagen accessible for *S. pneumoniae* adhesion (Kostrzynska and Wadström 1992).

1.4.2 Immune impairment and immune deviation

The bacterial infection is usually controlled and eliminated by multiple immune mechanisms during single infection. Following IAV infection however, this anti-bacterial response may be impaired. It was suggested that IAV infection can lead to

prolonged desensitization of TLRs for up to 6 weeks, resulting in augmented detection of and increased susceptibility to *S. pneumoniae* (Didierlaurent *et al.* 2008), but this increased susceptibility was only mentioned in the text of this paper and not shown in a data graph. Furthermore, the IAV induced apoptosis of cells results in the exposure of the inhibitory signal molecule CD200. CD200 can bind to its cognate receptor CD200R on myeloid cells, such as DCs and alveolar macrophages (Barclay *et al.* 2002), which results in reduced responsiveness of these myeloid cells to the bacteria (Goulding *et al.* 2011) (Figure 2B). This reduced ability to detect and respond to the bacteria means mice may control bacteria less well and succumb to disease.

Macrophages are important for the control and elimination of *S. pneumoniae* (Davis *et al.* 2011). These macrophages may become impaired following IAV due to reduction in numbers and decreased ability to phagocytose the bacteria. IAV specifically depletes the airway resident macrophages from the lung, which normally clear bacteria early in infection (Ghoneim *et al.* 2013). As these alveolar macrophages take up to 2 weeks to replenish (Ghoneim *et al.* 2013), there is a deficit of an early antibacterial response in the lung, thus facilitating an unchecked growth of the bacteria. Following co-infection the IFNγ production by T and NK cells provokes a decreased ability of any non-depleted macrophages to bind and phagocytose *S. pneumoniae*. IFNγ acts to downregulate the alveolar macrophage scavenger receptor MARCO resulting in decreased antibacterial function (Sun and Metzger 2008) (Figure 2C). The role of IFNγ in the impairment of the immune response during co-infection was confirmed in studies using linezolid treatment, which reduces IFNγ production. This treatment improved the survival of the hosts (Breslow-Deckman *et al.* 2013).

High concentrations of the antiviral cytokines, type I IFNs, can also compromise the immune response to the secondary bacterial infection by suppressing the anti-bacterial functions of macrophages and neutrophils (Shahangian *et al.* 2009; Li *et al.* 2012). Type I IFNs act to impair the recruitment of monocytes through suppression of the chemoattractant CCL2 during co-infection (Nakamura *et al.* 2011) (Figure 2D). Overall, the decreased ability of the macrophages to respond to infection may be increasing the susceptibility to secondary infection permitting a bacterial outgrowth.

Type I IFNs can also inhibit the recruitment of neutrophils by suppression of neutrophil chemoattractants such as CXCL1 and CXCL2 (Shahangian *et al.* 2009; Schliehe *et al.* 2014). Type I IFN production following IAV infection was found to induce Setdb2 protein, which correlated with repression of the CXCL1 gene. Setdb2 was found to have a negative effect upon early neutrophil recruitment, pathogen clearance and tissue integrity during IAV – *S. pneumoniae* co-infection. (Schliehe *et al.* 2014). Type I IFNs can also transiently reduce IL-17 production by γδT cells resulting in fewer recruited neutrophils (Li *et al.* 2012). Regardless of this effect by type I IFNs, it is found that neutrophil numbers are increased overall during co-infection (LeVine *et al.* 2001). This contradiction may be due to the outgrowth of bacteria driving the neutrophil recruitment which many override an initial IFN-induced impairment. Damjanovic and associates showed increased neutrophil numbers along with increased myeloperoxidase activity in the lungs of co-infected lungs mice, but with a marginally decreased phagocytic capacity *in vitro* (Damjanovic *et al.* 2013). In contrast, LeVine and others showed reduced myeloperoxidase activity of the neutrophils (LeVine *et al.* 2001). This discrepancy could be due to different IAV and *S. pneumoniae* strains, and the dosing used in

these two studies. The exact role of neutrophils during co-infection is not fully known. One study showed that antibiotic-induced lysis of bacteria resulted in an influx of neutrophils into the co-infected lungs, which caused extensive lung damage and mortality (Karlström *et al.* 2011). However this study also employed antibiotics that did not induce this influx of neutrophils and protected against mortality. The authors argue that the lysis of the bacterium by certain antibiotics elicits a 'proinflammatory burst', exacerbating disease (Karlström *et al.* 2011). Nonetheless this study along with the study by Schliehe *et al.* highlights the impact neutrophils can have on lung damage if they are recruited in high numbers.

Many studies have delved into whether neutrophils were protective during co-infection. Studies that depleted neutrophils during co-infection did not show exacerbated disease (Bogaert *et al.* 2010; Damjanovic *et al.* 2013). McNamee and colleagues showed that depletion of neutrophils when *S. pneumoniae* was given 3 days after IAV infection however did exacerbate disease, whereas no effect was seen when the pneumococcus was given 6 days post influenza (McNamee and Harmsen 2006). This study suggests that this is due to an increase in neutrophil impairment during IAV infection, thus they become non-protective late in infection. This study however depleted neutrophils with the low specificity RB6 antibody and also simultaneously treated IAV-infected mice with LPS aerosolization. This poor *in vivo* depletion and the potentially confounding LPS aerosolization make interpretation of these results difficult. It may be interpreted that neutrophil depletion is detrimental at 3 d.p.i due to the lower level of bacterial loads, whereas the effect of neutrophil depletion at 6 d.p.i cannot exacerbate disease further with the already high level of bacterial load within the lung.

Ellis and associates did describe a protective role for neutrophils. Blockade of neutrophils resulted in bacterial outgrowth and increased mortality (Ellis *et al.* 2015). Different timings and methods of infection along with different viral and bacterial strains may explain the differences between all these studies. It is therefore unclear whether the protective action of neutrophils is essential during co-infection and whether their impairment greatly exacerbates disease.

The immunosuppressive effects of type I IFNs induced by the primary infection are in contrast with studies that used IFNα as a treatment against co-infection. Treatment with this type I IFN increased the neutrophil and macrophage response, and survival of the mouse (Damjanovic *et al.* 2014). Overall it remains to be fully elucidated whether the role of type I IFNs during co-infections are beneficial or detrimental to the outcome of the response and infection. The timing and duration of IFN production may also make a difference to this outcome.

1.4.3 Enhanced proinflammatory responses

IAV and *S. pneumoniae* stimulate many of the same PRRs and induce similar effector molecules, therefore it is plausible that in the presence of both pathogens a synergistic response may occur. The apoptotic and inflammatory properties of the viral PB1-F2, may synergise with the pneumolysin of *S. pneumoniae* to induce exacerbated cell death and inflammation, leading to a ‘cytokine storm’ (McCullers 2014). Many studies have observed this synergistic increase in an inflammatory response even in the absence of PB1-F2 and pneumolysin, thus showing that this

cytokine storm may not solely be due to the synergism of these two pathogen proteins.

Increased TNF-α, IL-1β, CXCL1, CXCL2, IL-12 and IFN-γ have all been observed (LeVine *et al.* 2001; Seki *et al.* 2004; Smith *et al.* 2007; Damjanovic *et al.* 2013; Ellis *et al.* 2015). These increased proinflammatory cytokines and chemokines correlate with the increase in neutrophils mentioned previously (LeVine *et al.* 2001). As discussed, high concentrations of proinflammatory cytokines and chemokines may cause localised damage to the lung to the lung. These results appear to contradict the notion that an immune impairment is the mechanism for increased susceptibility to co-infection. It is possible that initial impairment in specific antibacterial function leads to bacterial outgrowth, which then drives an excessive immune response. Conversely, the combination of two pathogen triggers may lead to synergistic activation of immune cells or epithelia which in turn leads to massive immune cell recruitment, more cytokines and tissue damage. Which of these hypotheses is true is as yet unclear.

The responses of the airway epithelial cells, the first host targets of these two infections, have not been previously considered. The airway epithelial cells may contribute to the 'cytokine storm' and many of the other effects described in response to the combined stimulus. Inversely, the epithelial response may be impaired by initial exposure to IAV or IFNs. Delving into the role of the airway epithelium in co-infection will allow us to answer the question whether and by which mechanisms epithelial responses contribute to exacerbation of disease in this condition.

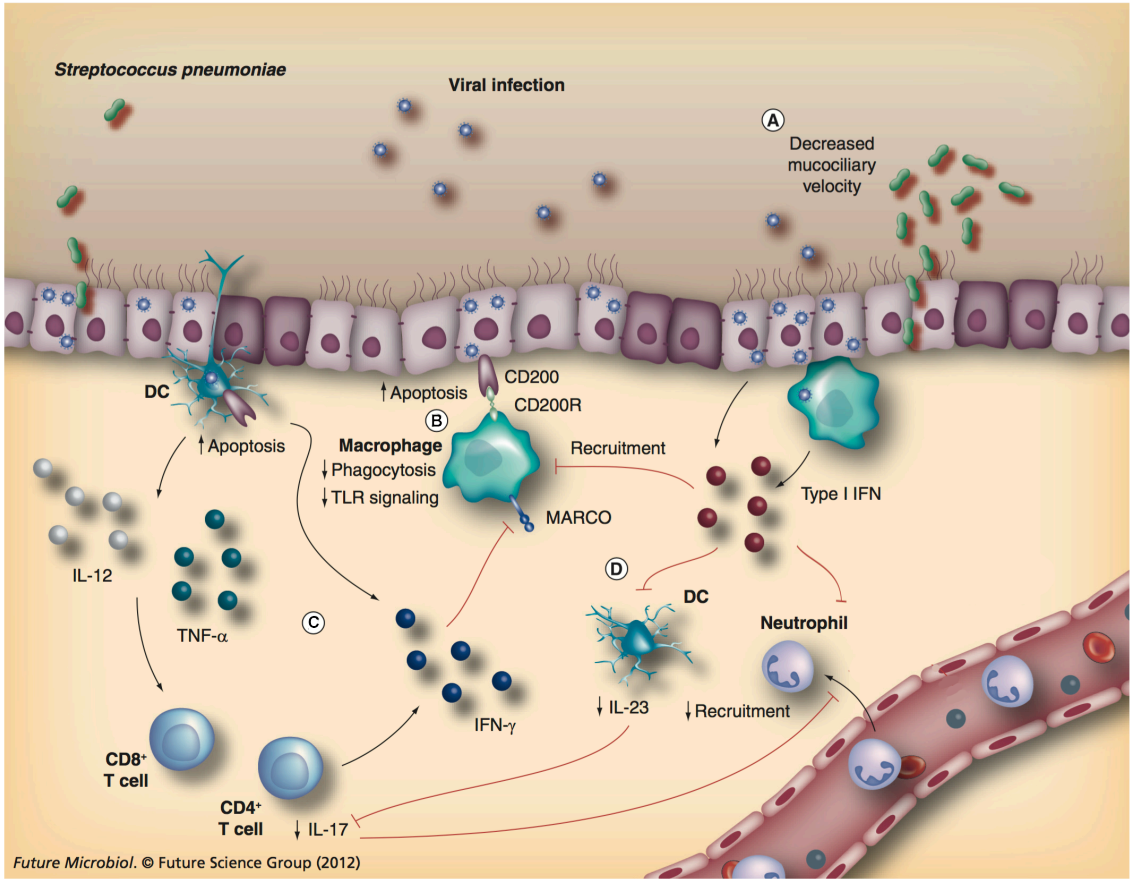


Figure 2 Immune response to IAV-*S. pneumoniae* co-infection

Viral replication within the airway epithelial cells decreases mucociliary velocity, thereby adversely affecting the clearance of *S. pneumoniae* (A). IAV infection also results in increased apoptosis of DCs and epithelial cells. Apoptotic cells increase their surface expression of CD200, which can subsequently bind to CD200R on myeloid cells, such as DCs and macrophages. Binding to the CD200R results in sustained desensitization to the bacteria (B). IAV infection induces the production of IFNγ by T-cells as well as endogenous IFNγ by APCs. IFNγ downregulates the scavenger receptor MARCO on alveolar macrophages, which leads to inhibition of their phagocytosis abilities of the bacteria (C). Production of type I IFNs following IAV infection can inhibit the recruitment of monocytes and neutrophils (D).
Figure adapted from (Short et al. 2012). Permission to reproduce this figure has been granted by Nature Publishing Group.

1.5 Host genetics and IAV

Differences in influenza severity or protection from influenza can be attributed to varying degrees of recall responses by the adaptive immune system to new and 'recognized' viral strains, depending on precedent vaccination or infection, or to the strain's virulence. However, apart from immunological memory there may also be a role for variations of host-intrinsic responses to the virus to explain differences in influenza severity (Horby *et al.* 2010).

Apart from host medical conditions such as disease- or pregnancy-related immunosuppression or risk factors for severe influenza such as heart defects, the host genetic background contributes prognosis of disease in influenza. Two studies delved into the role of familial aggregation in the susceptibility to mortality from influenza infection. Albright *et al.* utilized a database of 4855 influenza associated deaths in Utah between 1903 and 2004 (Albright *et al.* 2008). They found that genetically close (length of genetic path is less than 3) and distant relatives of individuals who succumbed to disease had a significantly increased risk of influenza-induced death themselves. A conflicting report by Gottfresson *et al.* concluded that of the 455 deaths from the 1918 'Spanish flu' in Iceland over a 6-week period, there was no statistically significant indication of a heritable predisposition to death (Gottfredsson *et al.* 2008). The Gottfresson *et al.* study however had a drastically lower cohort and a limited time period compared to the Albright *et al.* study. This would greatly impact on the generation of statistically significant outcomes. The Albright *et al.* study therefore is more reliable and as such indicates the contribution of a heritable component to the outcome of disease.

The more recent spread of the HPAI avian H5N1 has also highlighted the family clustering of infections and speculation that host genetics may therefore play

a role (Ungchusak *et al.* 2005; Kandun *et al.* 2006; Sedyaningsih *et al.* 2007; Wang *et al.* 2008). This is highly debated, with the possibility of this person-to-person transmission being attributed to close contact rather than host genetic factors (Pitzer *et al.* 2007). The totality of the epidemiological data rather supports the host genetic contribution to disease. In fact, up until 2010, 50 of the 54 H5N1 familial clusters were among blood relatives (Horby *et al.* 2010). A more recent study showed that of the 720 H5N1 infections reported since 1997 until 2nd November 2014, 144 cases were found in family clusters (55 clusters) (Qin *et al.* 2015). This study also identified 36 out of 460 cases of H7N9 infections were found in familial clusters (16 clusters). The increased proportion of family clusters found with H5N1 than with H7N9, could be due to a higher risk of infection by H5N1. The authors however exclude this, as there is a much faster rate of accumulation of human H7N9 cases compared to H5N1 cases. Another possible explanation is that the detection of H5N1 clusters is easier than H7N9 clusters. This explanation was ruled out due to the widespread availability of molecular diagnostic methods following the emergence of H7N9 in 2013. Thirdly, person-to-person transmission may be more common for H5N1 than with H7N9, but the authors found no evidence to support within their analysis. This leaves the final explanation of familial susceptibility to H5N1 as the only reasonable explanation for the higher incidences of clusters. This is corroborated by the overall relative risk of infection between blood-related contacts compared to unrelated individuals of 8.96 for H5N1 and 0.8 for H7N9. The authors however state that this relative rate of infection may be unrepresentative of the true number, as it could only be calculated where full data was available: 16 out of the 55 family clusters for H5N1, and 11 out of the 16 cluster for H7N1. They also suggest that a large number of mild cases of H7N9 infections go unreported, which

has also been reported by Yu *et al.* (Yu *et al.* 2013).

It is well established that the human leucocyte antigen (HLA) locus plays an essential role in inducing cell mediated lysis of IAV infected cells (McMichael *et al.* 1977). HLA also influences antigen presentation to T cells and drives specificity of the cytotoxic T lymphocyte (CTL) response to IAV (Belz *et al.* 2000; Boon *et al.* 2002). As of yet, no polymorphisms in the HLA loci have been found to associate with susceptibility to influenza infection.

Mutations in IRF7 (Ciancanelli *et al.* 2015), IFITM3 (Everitt *et al.* 2012; Wang *et al.* 2014), CD55 (Zhou *et al.* 2012), and the mutations and polymorphisms of TLR3 (Esposito *et al.* 2012), have all been associated with susceptibility to IAV. Polymorphisms in IL-1α, IL-1β, TNF and IL-6 genes have also been correlated with increased susceptibility to H1N1 infection (Liu, Li, *et al.* 2013; García-Ramírez *et al.* 2015). A study focusing on 91 severe 2009 ‘swine flu’ H1N1 cases and 98 exposed but asymptomatic controls found four single nucleotide polymorphisms (SNPs), which associated with disease severity (Zúñiga *et al.* 2012). Three of these SNPs were found within the genes for: complement binding protein (C1QBP), an immunoglobulin Fc receptor (FCGR2A) and a protein that facilitates entry of replication protein A into the nucleus (RPAIN). The final SNP is within an unknown gene: rs9856661.

HCV patients can also be classified according to SNPs in their IFNλ locus, where patients whose genome can code for the IFNλ4 protein have a reduced antiviral response to IFNα treatment and lower rates of spontaneous clearance of HCV, but with higher baseline ISG levels compared to individuals who do not have a functional IFNλ4 gene (Prokunina-Olsson *et al.* 2013). A negative feedback mechanism through prolonged signalling and induction of USP18 has been

hypothesised as the mechanism for which IFNλ4 antagonises other IFNs (Egli *et al.* 2014). As approximately 40% of Caucasians are thought to express IFNλ4, it would be of interest to assess if this SNP alters the IFNλ protectiveness in IAV infection.

The use of animal models has also indicated the genetic contribution to disease susceptibility. There have been several studies utilizing a range of inbred laboratory mouse strains to analyze their responses to IAV (Toth and Williams 1999; Boon *et al.* 2009; Srivastava *et al.* 2009; Alberts *et al.* 2010; Boon *et al.* 2011; Davidson *et al.* 2014). These studies showed that mice diverged in the outcome of disease dependent on their strain resulting in those that are susceptible and those that are resistant. Crossing a resistant strain with a susceptible strain resulted in intermediate levels of resistance (Srivastava *et al.* 2009), suggesting a codominant or polygenic contribution to influenza protection in these strains. Furthermore, some of these studies correlated increased proinflammatory responses to susceptibility (Boon *et al.* 2009; 2011). One study identified type I IFN directly correlated with exacerbated disease (Davidson *et al.* 2014). When Toth and Williams compared a resistant strain to a susceptible strain infected with a low pathogenic IAV strain (H3N2 X31), they found a quantitative trait locus (QTL) on chromosome 6 which associated with influenza-induced slow-wave sleep patterns (Toth and Williams 1999).

In 1962, the resistance of certain inbred mouse strains was reported (Lindenmann 1962) and was subsequently contributed to the Mx1 gene (Staeheli *et al.* 1988). Mx1 is able to inhibit influenza virus replication and protect the host during infection (Tumpey *et al.* 2007). Mice that are missing this gene or have nonsense point mutations are susceptible to IAV (Staeheli *et al.* 1988). The human homologue, MxA, along with the ISGs OAS1 and PKR, has been described and

associated with HCV outcome, but as of yet has not been associated with IAV severity (Knapp *et al.* 2003).

Host genetic contribution to IAV susceptibility has been well documented. However, the differences at the level of the airway epithelium have not yet been identified. The airway epithelium is the first host target of infection and the first to respond. Any defects in the ability of the epithelium to respond appropriately to infection may be detrimental for the host. The response of the epithelium from IAV-susceptible and resistant strains may therefore further clarify the contribution of host genetics to the outcome of IAV-induced disease.

1.6 Airway epithelial cells

1.6.1 Function

The respiratory system of all vertebrates has evolved to facilitate the vital exchange of gases between the organism and the outside environment. In humans, the branched structure of the airways allows for this bidirectional exchange of approximately six litres of air per minute (Rackley and Stripp 2012). The respiratory tract develops into a highly branched tree-like system. This design is highly conserved between vertebrates but can differ slightly due to the different sizes of the lungs (Rock *et al.* 2010). In general, the lung can be divided into two sections: upper respiratory tract and the lower respiratory tract (Figure 3).

The upper respiratory tract includes the nasopharynx, the trachea and the bronchi. The lower respiratory tract includes the bronchioles and the alveolar sacs. The proximal conducting airways are lined by a pseudostratified columnar epithelium whose main function is to transport air to and from the lower respiratory tract. It is also the first line of defence against any harmful contaminants within this inhaled air. This airway epithelium can act as a physical and a chemical barrier to pathogens by preventing the colonization and spread of the pathogen into the organism. The airway epithelium produces mucus, which traps and immobilises the pathogen, and the ciliated cells work in a coordinated wave of ciliar beats to mechanically move the mucus and therefore the pathogens out of the airways (Wolff 1986). This mucociliary transport is facilitated by the presence of two distinct layers of surface mucous. The layer closest to the airway epithelium is less viscous to allow for the free movement of cilia, and the overlying layer is more viscous to trap the particles and pathogens (Randell and Boucher 2006). Antimicrobial peptides are also produced and are secreted into these mucus layers to directly

destroy microbes. The epithelium monitors the external environment for these pathogens by using PRRs. If a pathogen is detected, the epithelium can release cytokines and chemokines for direct anti-pathogen action and to induce the recruitment and activation of the innate and adaptive immune response, to effectively eliminate the infection. These innate immune responses along with the tight adhesions between epithelial cells and the underlying stroma ensure that almost completely sterile, hydrated gases reach the peripheral alveoli for gas exchange. This defence function depends on the proper distribution and maintenance of the different epithelial cell types.

The alveolar epithelium of the lower lung allows for the essential process of gas exchange. The epithelium consists of type 1 and type 2 alveolar epithelial cells. The type 2 alveolar epithelial cells are cuboidal and contain abundant lamellar bodies, the secretory vesicles (Figure 3A). These cells secrete the surfactant-associated proteins (SPC-A –B –C -D) along with extracellular proteins and lipids which decrease the alveolar surface tension and contribute to host defence (Whitsett *et al.* 2010). These cells are also thought to contribute to the homeostasis of the lung through the absorption and secretion of fluid (Bove *et al.* 2010) and can act as progenitor cells for the other alveolar epithelial cells (Adamson and Bowden 1975). The type I alveolar cells are squamous or flat and constitute to approx. 95% of the surface area of the lower lung (Williams 2003). It is these type 1 alveolar cells that are essential for gas exchange (Figure 3B).

1.6.2 Structure of the upper respiratory epithelium

In mice, the largest airway is the trachea, which is lined with a pseudostratified columnar epithelium. This epithelium is composed of basal (Figure 3C), ciliated (Figure 3D) and secretory (goblet and club) cells (Figure 3E). It is these secretory and ciliated cells that make up the mucociliary defence mechanism described. This heterogeneous population also make up the human trachea, however mucin-secreting goblet cells are more abundant in humans than within the mouse. In humans, this pseudostratified epithelium can also extend into the bronchioles, whereas they are restricted to the trachea in mice (Mercer *et al.* 1994; Rock *et al.* 2010).

In both humans and mice the basal cells, so called due to their proximity to the underlying basal lamina, act as the stem cell of the lung capable of self-replenishing and differentiating into the two other cell types: ciliated and secretory. Basal cells constitute approximately 30% of the total epithelial population (Hong 2003; Hackett *et al.* 2008; Rock *et al.* 2009). They form a mostly continuous monolayer but can also be found in clusters or individually (Nakajima *et al.* 1998), and form desmosomal contacts with neighbouring columnar cells.

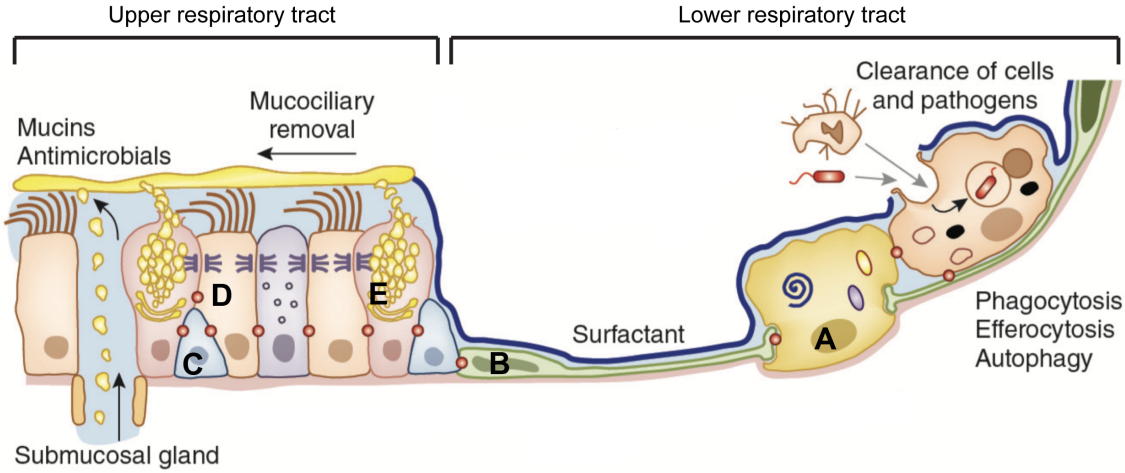


Figure 3 The cellular subsets of the upper and lower respiratory tract

The lung respiratory tract is split into two: upper respiratory tract and lower respiratory tract. The alveolar type 2 cells (A) secret surfactant to facilitate the gas exchange by the alveolar type 1 cells (B). The upper respiratory tract is made up of basal cells (C), ciliated cells (D) and secretory cells (E). The upper respiratory tract guides the inhaled air to the lower respiratory tract. The mucus production traps any inhaled particles. The cilia beat moves the mucus and trapped particles out of the airways.
Figure adapted from (Whitsett and Alenghat 2015). Permission to reproduce this figure has been granted by Nature publishing group.

1.6.3 Damage and regeneration

Epithelial cells of the lung are largely quiescent and turn over at a very slow rate, approximately every 100 days (Blenkinsopp 1967). Damage to the epithelium can be caused by bacterial or viral infections, harmful pollutants, inflammation, allergic reactions, physical trauma, cancer, or by unknown origins (idiopathic fibrosis). The epithelium must recover from this damage to return to homeostasis. The repair mechanisms are initiated immediately following damage to the epithelium with the proliferation of basal cells and progenitor cells which migrate and spread over the open wound. Once the denuded area is covered these stem cells differentiate into the missing cell types to re-establish the integrity and functional organization of the epithelial layer (Zahm *et al.* 1991; Puchelle *et al.* 2006; Rock *et al.* 2009).

Due to the difficulty of examining the *in vivo* repair mechanisms of the alveolar epithelium, less is known about it (Crosby and Waters 2010). Two studies however identified a ‘stem cell like’ population from the upper respiratory tract that migrates to the lower respiratory tract to repair the damaged epithelium, by covering the wound and differentiating into the alveolar cell types (Vaughan *et al.* 2014; Zuo *et al.* 2014). These stem cell like population express KRT5 protein and the transcription factor Trp63. These molecules are highly expressed in basal cells (Rock *et al.* 2009). Another study found that it was these cells that were preferentially infected following IAV infection, which significantly impacts the repair of the lower lung (Quantius *et al.* 2016).

The migration and spreading of the cells to cover the damaged area occurs within the first 12-24 hours following injury. The proliferation and differentiation of basal cells begins within 15-25 hours and continues for days or even weeks (Horiba and Fukuda 1994; Dupuit *et al.* 2000). Although basal cells are believed to

be the primary cell type capable of long-term self-renewal and differentiation, the club cells of the lung, which are normally quiescent, are believed to be able to dedifferentiate and also function as progenitor cells following damage, to replenish ciliated cells (Donnelly *et al.* 1982; Breuer *et al.* 1990; Watson *et al.* 2015). Once the ciliated cells are replenished, the club cells return to quiescence.

The damage to the epithelium and the ensuing inflammatory response results in the production of soluble factors from endothelial cells, fibroblasts, alveolar macrophages and the lung epithelium, which participate in the repair process (Crosby and Waters 2010; Herold *et al.* 2011). Of these soluble factors the epidermal growth factor (EGF) family and the fibroblast growth factor (FGF) family are believed to be important for epithelial repair. Several members of the EGF family have been shown to have roles in epithelial repair: EGF, transforming growth factor alpha (TGF-α), and amphiregulin (Kheradmand *et al.* 1994; Van Winkle *et al.* 1997; Monticelli *et al.* 2011). Innate lymphoid cells were found to produce amphiregulin in response to IAV infection to restore the airway epithelial integrity and tissue homeostasis (Monticelli *et al.* 2011). Three members of the FGF family have been attributed to epithelial repair in the lungs: keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and FGF-10 (Atabai *et al.* 2002; Crosby and Waters 2010; Herold *et al.* 2011). Administration of Fgf10 was found to counteract IAV-induced repair failure and restored barrier function in the lower lung (Quantius *et al.* 2016). Granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-22, although not conventional epithelial growth factors, have also been implicated in epithelial repair following IAV-induced lung damage (Pociask *et al.* 2013; Rösler and Herold 2016).

1.6.4 Airway remodelling

The ability of the epithelium to repair is extremely important, however an exaggerated response to chronic stimuli (Kim *et al.* 2008), repeated injury (Cao *et al.* 2016), or an aberrant response to naïve stimuli may result in airway remodelling. The immune response to infections has also been shown to induce airway remodelling, with some cytokines altering the cell fate choices, contributing to goblet cell metaplasia (Danahay *et al.* 2015). Loss of growth factors, such as amphiregulin, or cells which produce these factors can also result in airway remodelling (Monticelli *et al.* 2011). This remodelling results in variations in tissue architecture, cell composition and matrix properties, which may affect the pulmonary mechanisms and gas exchange capacity of the lung (Crosby and Waters 2010). Pathological changes in the cellular composition (remodelling) and physiological function of the airways are common features of respiratory diseases, which include chronic asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and idiopathic pulmonary fibrosis (IPF) (Thorley and Tetley 2007; Crosby and Waters 2010; Rock and Hogan 2011).

Asthma

Asthma is a common long-term disorder of the lung. Hallmarks of asthma include goblet cell hyperplasia and overproduction of mucus along with repeated epithelial shedding, airway wall fibrosis, thickening of the basal lamina, angiogenesis, and hyperplasia of the submucosal gland (Tang *et al.* 2006). Asthma suffers also experience exacerbations of virus induced disease (Kurai *et al.* 2013). Asthma is believed to be partly caused by allergen-specific T helper 2 (Th2) cells which produce the cytokines: IL-4, IL-5, IL-9, IL-10 and IL-13 (Cohn *et al.* 2004). Induction

of these cytokines, apart from IL-10, correlates with airway inflammation, including eosinophilia, mast cell activation and goblet cell metaplasia (Lee *et al.* 1997; Temann *et al.* 1997; Curran and Cohn 2010). It is also thought that a defect in epithelial repair may result in this airway remodelling. This defect is proposed to be present from birth as these clinical manifestations have been observed early in life (Pohunek *et al.* 2005). One study has shown that epithelial growth factor receptor (EGFR) may be altered in asthma and thus affect the repair capabilities of the epithelia (Puddicombe *et al.* 2000). Basal cells are present in the areas of goblet cell hyperplasia (Rock *et al.* 2010), therefore it is possible that changes in their ability to proliferate and differentiate may contribute to the ability of the lung to repair.

COPD

COPD is a major cause of morbidity and mortality, with around 6% of men and 4% of women suffering from COPD in the UK (Barnes 1998). It is predicted by the World Health Organization (WHO) to become the third leading cause of death worldwide by 2030 (Rock *et al.* 2010). Patients suffering from COPD experience disease exacerbation and death following influenza infection (Bauer *et al.* 2010; Kurai *et al.* 2013), and even more so in co-infection (Sethi 2006). Smoking is one of the most important causative factors in the development of COPD, inducing chronic bronchitis, small airways disease, and emphysema (Thorley and Tetley 2007; Kang *et al.* 2008), leading to narrowing of the airways, shortness of breath (dyspnea) and persistent cough that brings up thickened, discoloured mucus. The airway epithelium is the first target of cigarette smoke and other inhaled toxins. The epithelium adapts to protect the lung from theses toxins, however in those that

develop COPD the changes within the epithelium become irreversible affecting the structure and function of the lung. Patients afflicted with COPD also have a higher risk of developing lung cancer than smokers without COPD (Mannino *et al.* 2003). This progression is thought to start with epithelial hyperplasia, turning to metaplasia, to dysplasia, carcinoma in situ and subsequent malignancy (Banerjee 2009; Rock *et al.* 2010). Again changes in basal cell proliferation and differentiation capabilities may induce this remodelled state of the lung. Hyperproliferating basal cells were observed in these metaplasia areas in COPD patients, which were found to produce IL-1β. This IL-1β production promotes airway wall fibrosis (Araya *et al.* 2007). Although IL-13 is synonymous with Th2-mediated allergy, causing asthma, IL-13 driven chronic lung disease has also been implicated in COPD following viral infections of the lung (Kim *et al.* 2008).

Cystic fibrosis

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR). A disrupted ion transport within the columnar epithelial cells is the primary defect within the lungs of these patients (Boucher 2007). Along with the resulting dehydration of the lung, chronic bacterial infection, goblet cell hyperplasia, impaired mucin clearance, increased proinflammatory cytokine secretion, extensive leukocyte influx, airway epithelial damage and bronchiectasis are also observed (Rock *et al.* 2010). Furthermore, hyperproliferative basal cells were observed in these patients, contributing to the chronic repair process (Voynow *et al.* 2005). These chronic infections, repeated damage, and repair of the lung change the overall lung cellular composition and function. In turn, this altered lung architecture facilitates further infections.

Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a type of interstitial lung disease (ILD). IPF is a severely debilitating chronic and ultimately fatal lung disease. Idiopathic means the cause of this condition is unknown. It is generally thought that chronic epithelial injury and disrupted repair leads to disordered communication between the epithelium and the surrounding stroma. This leads to mesenchymal hyperproliferation and fibrogenesis of the lung (Pardo and Selman 2002). It is currently unknown which cell types are the fibrogenic population, although resident fibroblasts, bone marrow-derived stem cells and alveolar epithelial cells have been proposed (Willis *et al.* 2006; Rock and Hogan 2011). Again this altered lung architecture leaves the host vulnerable to infections, which induce further damage, and induction of repair mechanisms resulting in fibrosis and loss of lung function.

It is clear that chronic infections and repair leads to severe lung disease and remodelling leaving the host vulnerable to subsequent infections, thus resulting in a vicious circle. Changes to the basal cell proliferative rate or disruption in the amount of ciliated and secretory cells could theoretically lead to pathological airway remodelling. Excessive basal cell proliferation could lead to basal cell hyperplasia at the expense of differentiation. On the other hand, inappropriate cell fate choices may lead to goblet cell hyperplasia or ciliopathies at the expense of ciliated cells. Ciliopathies comprise a group of disorders where a lack of, or abnormal formation or function of ciliated cells are observed (Waters and Beales 2011). A failure to proliferate, increased apoptosis along with this inappropriate commitment to

differentiation could also contribute to epithelial remodelling (Rock *et al.* 2010). In summary it is clear that in many cases repeated repair can cause considerable changes to the structure and function of the lung. Therefore, in the airway epithelium, a tight balance of the basal cell proliferation and differentiation is required, and any change in the ability of basal cell proliferation or differentiation may impact severity and long-term outcome of infection.

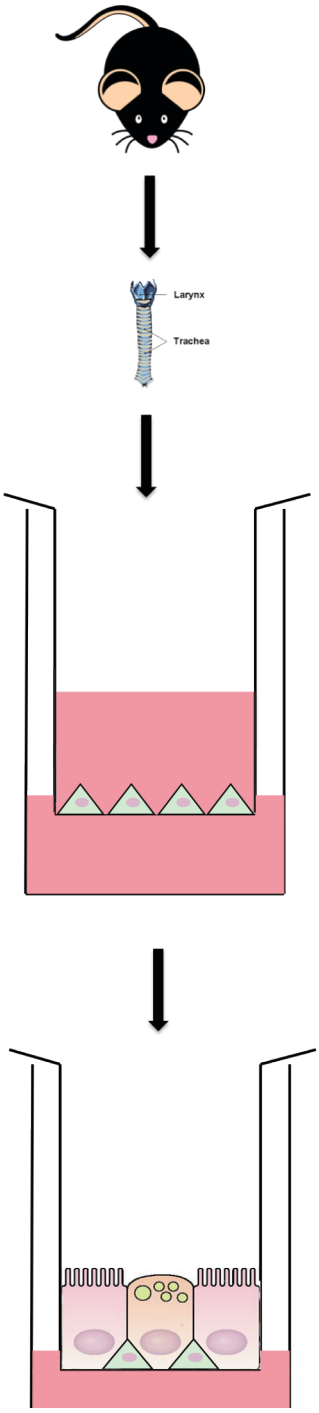
1.6.5 Mouse tracheal epithelial cells

Most pathogens infect the upper respiratory tract and as such the mouse trachea becomes a highly useful experimental model. The mouse trachea is very similar to the human trachea, bronchi, and bronchioles. Therefore, the culture of primary mouse tracheal epithelial cells (mTEC) provides a potent tool for the study of the composition and response of airway epithelial cells (AECs) in respiratory tract development and during infections. Utilising this primary culture also allows for the generation of genetically different epithelial cells from different inbred strains of mice and genetically altered mice, to determine the contribution of host genetics to the development and response of the AECs.

To generate these mTEC cultures, epithelial cells are digested out of the mouse trachea and plated on transwells in the presence of a growth medium. Only a fraction of the seeded cells attach to the transwell membrane as differentiated cells die, leaving a few remaining basal cells, which proliferate until they are confluent. The media from the upper chamber of the transwell is then removed to expose the basal cells to air. This air liquid interphase (ALI) stimulates the basal

cells to differentiate into secretory and ciliated cells (Ostrowski and Nettesheim 2009) (Figure 4). The full growth and differentiation of this culture takes about 14 days (You *et al.* 2002).

Using these mTECs will allow us to focus on the contribution of epithelial cells in infectious settings as outlined previously. We will be able to study epithelial responses during viral-bacterial stimulation, the effect of host genetics on the response to IAV. mTECs will also allow us to determine the effectiveness of a therapeutic treatment against IAV, and the impacts of IFNs during repair.



Digest epithelial cells out of mouse trachea

Plate on transwell

Grow to confluence
(R> 1000 mΩ/cm^2)

Remove medium from upper chamber
(air - liquid interphase, ALI)

Differentiation into airway epithelium

Figure 4 Generation of an mTEC culture

Epithelial cells are digested out of a mouse trachea and seeded onto a semi-permeable transwell along with growth medium. The cells are grown to confluence (trans-epithelial electric resistance (TEER) >1000mΩ/cm^2). The upper medium is removed from the upper chamber to expose the cells to air (air liquid interphase (ALI)). This exposure to air induces the differentiation of the cells to resemble the tracheal airway epithelium.

1.7 Study Rationale

We aim to study the protective and pathological processes related to influenza infection from a strictly airway epithelial perspective. The airway epithelium represents the first host target of many infections and is ultimately the first line of host defence, responding by producing cytokines and chemokines to modulate the immune response and repair the damage induced by any harmful particles inhaled. Severe IAV induced disease is characterised by a 'cytokine storm' and lung damage. It is intriguing to note that in studies of severe influenza, mortality does not correlate with viral load but with the magnitude of the immune activation (Hayden *et al.* 1998; Peiris *et al.* 2004). IAV infection can induce morbidity and mortality of the host, however a number of factors can determine disease outcome, such as secondary bacterial infections, host genetics and disrupted repair. The airway epithelium response to infection may therefore play a critical role in outcome of disease.

Secondary bacterial infections represent the majority of deaths following IAV pandemics (Madhi *et al.* 2004; Thorburn *et al.* 2006; Morens *et al.* 2008). This trend is also common in seasonal IAV epidemics (O'Brien *et al.* 1996; Metersky *et al.* 2012). Research into the immune response to IAV-*S. pneumoniae* co-infection has been extensively undertaken, however several aspects remain elusive. The massive proinflammatory response is well documented in co-infected lungs (LeVine *et al.* 2001; Seki *et al.* 2004; Smith *et al.* 2007; Damjanovic *et al.* 2013; Ellis *et al.* 2015). The primary and main producers of these proinflammatory cytokines remains unidentified. As the airway epithelium is the first host target of viral infection and the first cell type exposed to invading bacteria, and can respond by producing proinflammatory cytokines, we have utilised the mTEC system to

determine the AECs response to viral and bacterial stimuli both separately and together. Using this system allows us to determine if the AECs can orchestrate a synergistic response to co-infection, which may contribute to, if not be the sole responsible determinant of the increased morbidity and mortality observed in co-infections.

Type I IFNs are the prototypical antiviral cytokines and are thought to be exclusively protective during IAV infection. Work within our lab has however shown that a high IFN response correlates with increased disease severity and lung damage (Davidson *et al.* 2014). It is intriguing that in different in-bred mouse strains a divergent response to IAV infection occurs, which may be an indicator of a differing epithelial makeup and response. We hypothesise that the airway epithelium from the high IFN, IAV-susceptible mouse strain is driving the high proinflammatory response seen *in vivo*, and will also have reduced potential for repair following injury.

Owing to our study on the pathogenic potential of type I IFNs (Davidson *et al.* 2014), we then assessed the potential of type I IFNs (IFNα) and type III IFNs (IFNλ) as a treatment for IAV infection. Isolation and stimulation of both mouse and human cell types and epithelial cells, will allow for the determination of the cell specific effect of these IFNs. We hypothesise that owing to the expression of the IFNλ receptor on airway epithelial cells only, IFNλ will be a better treatment option than IFNα against IAV, as it will not induce the immunopathology seen with IFNα.

As discussed, the presence of high type I IFNs can be detrimental during IAV infection whereas IFNλ is beneficial. However, what effect these IFNs can have when IAV infection is cleared and the lung epithelia are repairing has not been fully investigated. The antiproliferative effect of type I IFNs is well known (Paucker *et al.*

1963; Bekisz *et al.* 2010), whereas the antiproliferative effects of type III IFNs are only recently being appreciated (Brand 2005; Maher *et al.* 2014). The effect of type I and type III IFNs have not been assessed on repairing epithelia of the lung. We hypothesise that IFNs will have a negative effect on proliferation and differentiation. We aim to determine which mechanism is behind this antiproliferative effect. We will utilise both type I and III IFNs to establish which IFNs are eliciting this effect.

In conclusion, the following 4 questions will be addressed in this thesis:

1. Do airway epithelial responses to combined viral-bacterial stimuli explain or contribute to the severe immunopathology observed in IAV-*S. pneumoniae* co-infection?
2. Do the host genetic differences in anti-IAV responses observed in C57BL/6 versus 129 mice manifest in airway epithelia?
3. How do the different response patterns of immune and epithelial cells to IFNα versus IFNλ explain their different therapeutic potential in IAV infection?
4. How do IFNs impact on airway epithelial repair?

Chapter 2. Materials & Methods

Mice

129S8 and C57BL/6 mice were bred at the Francis Crick Institute, Mill Hill Laboratory under specific pathogen-free conditions. B6.A2G-Mx1 congenic mice carrying the functional Mx1 alleles on the C57BL/6 background (Staeheli *et al.* 1985) were a kind gift from Dr P. Staeheli (Freiburg University). All mice were kept in specific pathogen-free isolators until use for experiments. Clinical symptoms during influenza infection were scored based upon presentation of hunched posture, piloerection, reduced movement and labored breathing. All protocols for experiments with animals were approved by the Home Office, UK, Animals (Scientific Procedures) Act 1986.

Primary mouse tracheal epithelial cell culture (mTEC)

Isolation and culture of primary mTEC were performed as previously described (You *et al.* 2002). In summary, tracheas from mice were obtained and cells isolated by enzymatic treatment were seeded onto 0.4 mm pore size clear polyester membrane (Corning) coated with a collagen solution. At confluence, media was removed from the upper chamber of the transwell to establish an air-liquid interface (ALI). Fully differentiated, 7-10 days-old post ALI cultures were used for experiments. For replated experiments the cells were dissociated from the transwells using Trypsin-EDTA (0.25%) (ThermoFisher) supplemented with 10mM EDTA (ThermoFisher). The cells were washed and resuspended in growth medium. The cells were counted and then plated as before in or without the presence of IFNs. The time taken to reach confluence (R>1000 $m\Omega/cm^2$) was recorded. MTEC cultures will also be referred to as airway epithelial cells (AEC)

throughout this thesis.

A549 culture

Human lung carcinoma epithelial-like cell line A549 was obtained from Dr. J. McCauley (Francis Crick Institute, Mill Hill Labs (FCI-MH)) and cultured in RPMI medium supplemented with 10% fetal bovine serum in a T75 culture flask at 37°C. At approximately 70-95% confluency cells were detached from the flask by Tryspin-EDTA treatment, pelleted and reseeded at 1 x 10^6 cells/well, in a 24-well plate

Influenza Virus

Influenza A virus X31 strain (H3N2), a reassortant with the A/PR/8/34 (H1N1) backbone, and PR8 (A/PR/8/34, H1N1), kind gifts from Dr. J. Skehel (FCI-MH), were grown in 10 day embryonated chicken eggs which are free of bacterial, mycoplasma, and endotoxin contamination. Alternatively, the virus was grown in Madin-Darby Canine Kidney (MDCK) cells, a kind gift from Dr J. McCauley (FCI-MH). All viruses were stored at -70°C and titrated on MDCK cells by 50% tissue culture infective dose ($TCID_{50}$), according to the Spearman-Karber method.

Virus infection in vitro

The apical sides of the mTECs were washed extensively to remove accumulated mucus before inoculation with X31 at a MOI of 0.1. After incubation at 37°C for 4 hours, the virus inoculum was removed. Cells were then lysed to extract RNA. For the A549 cells the media was removed and cells were inoculated with IAV at a MOI of 0.3 in media without sera. After 4 hours the inoculum was removed and complete media re-added. The cells were lysed after 24 or 48 hours.

Virus infection and treatment in vivo

Mice were anesthetized by inhalation of isofluorane and infected via the intranasal route with X31 ($8x10^2$-$2.4x10^4$ $TCID_{50}$/30μl) diluted in phosphate-buffered saline (PBS). Virus quantification was carried out by quantitative real-time PCR (RT-qPCR) on complementary deoxyribonucleic acid (cDNA) for the matrix gene, as previously described (Ward *et al.* 2004). In Chapter 4, C57BL/6 mice were infected with X31 as described and treated with EdU (1 mg/mouse) (ThermoFisher) or PBS on days 7-9 post infection. Lungs were collected on day 10 post infection. In Chapter 5, B6.A2G-Mx mice were infected with PR8 ($3x10^4$-$1x10^5$ $TCID_{50}$/30μl). B6.A2G-Mx mice were treated with 1.45μg/50μL of IFNα or 2.6μg/50μL IFNλ either at -1dpi (pre-treatment experiment) or days 2, 4 and 5 post infection (treatment during infection experiments). Mice were infected and treated under light isoflurane-induced anaesthesia intranasally. All anaesthesia was performed with animals kept on a heat mat to regulate body temperature.

Streptococcus pneumoniae

D39, a kind gift from Dr. M. Coles, (University of York) was grown from cryopreservative beads (Technical Services Consultants) in brain-heart infusion broth under microaerophillic conditions to autolytic phase overnight, then subcultured to an optical density of 0.4 at OD 590/600, centrifuged and re-suspended in PBS immediately prior to infection both *in vivo* and *in vitro*. To heat kill the bacteria, it was placed at 80°C for 10 minutes, and killing of bacteria confirmed by plating. TIGR4, a kind gift from Dr. K. Okkenhaug (Babraham), was passaged through mice. Colonies were collected from spleens of infected mice and incubated in brain-heart infusion broth under microerophilic conditions to autolytic

phase overnight, then sub-cultured in brain-heart infusion broth to an optical density of 0.5-0.7 (OD600) before being centrifuged and re-suspended in PBS + 10% glycerol to an optical density of 1.0 (OD600). Aliquots were snap frozen and stored at -70^{o}C for a maximum of 10 months before re-passage through mice.

Bacterial infection

For *in vitro* co-exposure experiments, 24 hours after primary virus infection, the apical side of the cells were washed extensively to remove accumulated mucins before inoculation with live or heat killed (HK) *S. pneumoniae* at 10^6 CFU/well, or TIGR4 at 10^8 CFU/well. After incubation at 37°C for 23.5h, the antibiotic: penicillin streptomycin was added and aliquots of the supernatants were collected before and after for plating on blood agar plates to determine growth and subsequent death of bacteria. Cells were then lysed to extract RNA 24h post bacterial inoculation. For *in vivo* co-infection experiments, mice were anesthetized by inhalation of isofluorane and infected via the intranasal route with 30µl of the bacteria diluted in PBS.

Toll like receptors

Toll-like receptor 2 agonist, Pam3CSK4 (Enzo) and toll-like receptor 4 agonist, LPS (Enzo) was given apically to the AEC cultures 24 hours after IAV infection. Cells were then lysed to extract RNA 24h post TLR agonist treatment.

Recombinant IFN proteins

IFNα: Mammalian IFNα4 for mouse studies (Lot: 5983) and human universal type I IFN (5831) was purchased from PBL Assay Science (Chapter 5 and 6)

IFNβ: Mouse IFNβ was purchased from PBL Interferon source (Lot: 5118) (Chapter 4 and 6).

IFNλ: A codon optimized cDNA encoding the mature form (without the signal peptide) of mouse IFNλ2 was purchased (Eurofins) and expressed in E. Coli, purified under denaturizing condition and refolded *in vitro* as described previously (Dellgren *et al.* 2009), a kind gift from Dr R. Hartmann (Aarhus University). The human IFNλ3 was made as described in (Dellgren *et al.* 2009) (Chapter 5). For Chapter 4 and 6, IFNλ2 (Lot: 4635-ML) and IFNλ3 (Lot: 1789-ML) were used jointly as the IFNλ stimulus and were purchased from R&D systems.

IFN titration on AECs and subsequent generation of an IFNα:IFNλ conversion ratio

mTEC cultures were stimulated for 4hrs with serial dilutions of IFNα, IFNβ or IFNλ or medium control. For Chapter 5 mouse IFNα was compared to mouse IFNλ with a serial dilution ranging from ranging from 100 – 3 U/ml IFNα vs. 1-0.03 ng/ml IFNλ, and 100 – 0.16 U/ml human IFNα vs. 100 -0.03 ng/ml human IFNλ. In Chapter 6, IFNα and IFNβ were compared using the range 100 - 3 U/ml, and IFNβ and IFNλ were compared using 30 – 0.24 (U/ml and ng/ml respectively). The induction of stated ISGs was assessed by RT-qPCR. For each gene, data was pooled from 2-8 independent titrations. Prism 6 software was used for four-parameter logistic regression analysis, to generate a dose response curve and obtain half-maximal effective concentrations (EC_{50}) for each gene assessed for each treatment. An IFNα:IFNλ (Chapter 5), and IFNα:IFNβ:IFNλ (Chapter 6) conversion ratio was then generated by for example: dividing the IFNα EC_{50} for an ISG by the IFNλ EC_{50} for the same gene (Chapter 5). The final conversion ratio of 0.558 was determined by the geometric mean of the ratios obtained for all ISGs assessed and applied to

treat mice and cells with equipotent amounts of IFNα and IFNλ (Chapter 5). The conversion ratio for human IFNα:IFNλ of 17.5 was applied to the human cells (Chapter 5). For Chapter 6, this conversion ratio was 2:1:1 IFNα:IFNβ:IFNλ (U/ml:U/ml:ng/ml).

Stimulation of mouse cells

In Chapter 4, equipotent doses of 30 U/ml of IFNβ and 30 ng/ml IFNλ were added to the upper and basalolateral side of the mTEC cultures for 4 hours. The cells were washed and lysed for analysis.

In Chapter 5, equipotent doses of IFNα and IFNλ were added to the apical and basolateral sides of the cultures for 4 hours. The cells were then lysed for analysis. Spleens were excised from B6.A2G-Mx1 mice. Spleens were then were directly mashed through a 70μM cell strainer and washed with flow cytometry buffer. Red blood cells were lysed using ammonium chloride and cells were seeded into a 96-well U-bottom plate at 1x106/well. Whole splenocyte cultures were stimulated with IFNα4 (100 U/ml), IFNλ2 (1.4 ng/ml) or left as media control for specified time points. After stimulation cells were collected for analysis by RT-qPCR and multiplex.

In Chapter 6, AECs were grown in the presence of IFNα, IFNβ, IFNλ or media control ranging from 270 – 5 U/ml (IFNα and IFNβ) and 270 – 5 ng/ml (IFNλ), throughout the full term of culture.

IFN Stimulation of human cells

The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents. Primary human bronchial

epithelial cells were purchased from Lonza and cultured as per manufacture's instructions. In brief, cells were expanded in a T-75 flask to 60% confluence then harvested for seeding onto transwells at 50,000 cells per insert. At confluence, liquid was removed from the upper chamber to establish ALI. Fully differentiated, 15-20 day-old post ALI cultures were routinely used for experiments. Peripheral blood mononuclear cells (PBMCs) were prepared from peripheral blood by Ficoll-Paque density gradient centrifugation, and cultured at $2x10^5$ cells per well. For analysis of cytokine secretion, primary human bronchial epithelial cell and PBMC cultures were stimulated with human universal type I IFN (21 U/ml) and human IFNλ (1.2 ng/ml) or media controls for 24hrs.

Separation of CD45+ and CD45- cells from whole lung

Whole lungs were excised and processed into single cell suspension using gentleMACS (Miltenyi), following the manufacturer's instructions. Cells were then incubated with biotin-conjugated CD45 (Biolegend) for 15 mins at 4^oC in MACs buffer (2mM EDTA, 2% FCS in PBS). Cells were washed x1 with MACS buffer and incubated with anti-biotin microbeads (Miltenyi) in MACS buffer for 15 mins at 4^oC. Cells were washed, suspended in MACs buffer and separated by MACS cell separation (LS columns), as per manufacturer's instructions. The CD45+ and CD45- fractions were collected and processed for RNA extraction.

RNA extraction

Before RNA extraction, mTEC cultures were washed extensively with PBS. RNA was isolated from MTEC cultures by directly lysing the cells in the transwells, using the Qiagen RNeasy mini kit, according to the manufacturer's instructions. Whole

lungs were collected in TRIzol (Invitrogen) and homogenized using Polutron PT 10-35 GT (Kinematica). One microgram total RNA was reverse transcribed using the ThermoScript RT-PCR System kit (Invitrogen). The cDNA served as template for the amplification of genes of interest and the housekeeping gene (Hprt1) by real-time PCR, using TaqMan Gene Expression Assays (Applied Biosystems), universal PCR Master Mix (Applied Biosystems) and the ABI-PRISM 7900 sequence detection system (Applied Biosystems). The increase in mRNA expression was determined using the ΔC_t method. The fold increase in mRNA expression was determined using the $\Delta\Delta C_t$ method relatively to the values in mock treated samples, after normalization to Hprt1 gene expression.

Scratch Assay

After the mTEC culture was fully grown (d14 after plating), a pipette tip (size p2) was used to induce a 'scratch' transversely across the mTEC culture. The transepithelial electrical resistance (TEER) was measured until the culture reaches back to confluence and the scratch is considered repaired. To quantify the proliferative rate of the cells following a scratch, the mTEC cultures were pulsed with EdU for 24 or 48 hours then taken for quantification by Flow Cytometry.

Immunohistology

For staining of cilia in the mTEC cultures, cells were fixed within the transwell with 4% paraformaldehyde in PBS for 60 mins. Cells were then washed with PBS, then treated with 0.1% Triton-X (Roche) in PBS for 15 mins, washed again with PBS and blocked with 0.5% BSA for 30 mins. β-tubulin (Sigma) was added in PBS/BSA for 1 hour. Cells were washed for 5 mins in PBS x3. The cells were then treated

with anti-mouse Alexa 488 for 1 hour in PBS/BSA and cells were once again washed for 5 mins in PBS x3. Cells were then stained with DAPI for 10 mins and washed. The transwell membrane is cut out and placed on a glass slide, covered with a mounting medium (Vectashield, Vector Laboratories) and a coverslip. Tracheas were excised, mounted in paraffin and cut into slices and mounted on glass slides. To process the samples for staining, the wax was melted and washed with xylene, and then in decreasing concentrations of alcohol to rehydrate the samples. To expose the antigens, the glass slides were heated in 10 mM NaCit and stained as with the mTEC cultures. All slides were run on a VS120 virtual slide microscope.

Microarray data analysis

RNA was hybridized to Illumina.SingleColor.Mouse WG-6_V2_0_R0_1127 microarrays. Raw data were processed using GeneSpring GX version 11.5 (Agilent Technologies). After background subtraction, each probe was attributed a flag to denote its signal intensity detection P-value. Flags were used to filter out probe sets that did not result in a 'present' or 'marginal' call in at least 50% of the samples, in any one out of three experimental conditions. The signal intensity of each probe was first normalized on the median intensity of that probe across the control group and then represented as log2/log4 fold change relative to the controls. Subsequent statistical analysis was a 1-way ANOVA to identify genes significantly differentially expressed relative to controls (≥2 fold change; $P<0.01$, Benjamini-Hochberg multiple test correction) which were further analysed by a further 2 fold change to identify genes significantly differentially expressed in co-exposure versus single exposure (Chapter 3), or IFN treatment (Chapter 6). The

subset of genes most differentially expressed were analysed by Ingenuity Pathway Analysis.

Quantification of protein

Bronchioalveolar lavage (BAL) fluid was recovered from treated mice, centrifuged at 1,300rpm, 5min at 4^{o}C and supernatant collected. Cell culture supernatants were harvested from the apical side of the cultures. IL-28A/B was measured using the IL-28A/B ELISA Duo kit (R&D Systems), IFNβ and IFNα with their respective Verikine ELISA kit (PBL Interferon Source). CXCL1 and CXCL2 with their respective ELISA Duo kit (R&D Systems), IL-6 and TNF-α were measured with their respective ELISA Ready-SET-Go!® (eBioscience). Milliplex Map Kit was used to quantify the protein of samples as per manufacturer's instructions and read on a Luminex 100 (E-Bioscience). For immunoblots of total lysates, mTECs were lysed in RIPA buffer (Appendix, Table 1). Detection of p-EGFR (Cell Signaling Technology), T-EGFR (ThermoFisher) and β-actin (Cell Signaling Technology) protein by western blot and quantification of the protein was undertaken by ImageJ analysis.

Flow cytometry

mTECs: The different cell subsets of mTECs were quantified by flow cytometry. In brief, mTECs were dissociated from the transwells using Trypsin-EDTA. The cells were pelleted and washed with PBS x2. Cells were preincubated with anti-FcγRIII/II (Fc block) in PBS prior to a 20 min incubation with the fluorochrome-labelled antibodies (Appendix, Table 2). Cells were then washed with PBS x2 counter stained with LIVE/DEAD® Fixable Dead Cell Stain (Life Technologies) for 15 mins.

Whole lungs: Lungs were excised from infected and EdU treated mice or untreated

mice, digested with 20µg/ml Liberase TL (Roche) and 50µg/ml DNAse 1 (30 minutes at 37°C) and homogenized using gentleMACS (Miltenyi), following the manufacturer's instructions. Lungs were then passed through a 70µM cell strainer and washed with flow cytometry buffer (10% BSA in PBS). Red blood cells were lysed using ammonium chloride and cells were seeded into a 96-well U-bottom plate at $1x10^6$ cells/well. Cells were preincubated with anti-FcγRIII/II (Fc block) in PBS prior to a 20 min incubation with the fluorochrome-labelled antibodies (Appendix, Table 3). Cells were then washed with PBS x2 counter stained with LIVE/DEAD® Fixable Dead Cell Stain (Life Technologies) for 15 mins. For intracellular staining of EdU, the Click-iT® EdU Alexa Fluor® 488 (ThermoFisher) imaging protocol was followed. All samples were resuspended in the PBS buffer (or permeabilization buffer if intracellular staining) and analyzed using a LSR II or BD LSRFortessa X-20 (Becton Dickinson).

Statistical analysis

Data shown as the means ± SEM. Data sets were analysed by 2-way ANOVA, 2-way ANOVA with Bonferroni post tests, Log-rank (Mantel-Cox) Test (survival) or Student-t test. GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used for data analysis and preparation of graphs. P-values less than 0.05 were considered as statistically significant

Chapter 3. IAV and *S. pneumoniae* show limited synergy in inducing epithelial responses

3.1 Background

A major complication following IAV infection is the increased susceptibility of the host to infection with bacteria such as *Streptococcus pneumoniae.* Combination of these divergent stimuli synergizes leading to increased induction of lung damage, morbidity and mortality (McCullers and Rehg 2002; Brundage and Shanks 2008). It is not fully understood what the cause of this increased lung damage, morbidity and mortality is.

The lung relies heavily on the innate immune response to protect the host from invading pathogens. *S. pneumoniae* must be able to evade the host response mechanisms to infect the host. Additionally, the primary viral infection may predispose the host to a more severe manifestation of the secondary bacterial infection by direct viral-mediated lung damage, impairment of the antibacterial immune response, or induction of immunopathology. Many of the innate immune responses to co-infections have been analysed *in vivo*. However, the specific role of the airway epithelium in these responses has not been previously considered. The airway epithelium is at the forefront of the host's defence since it acts as a physical barrier to infection and is the first to respond. It can produce antiviral and antibacterial effector proteins, and cytokines and chemokines to recruit and activate immune cells, contributing to the elimination of the invading pathogens. With regard to the epithelial response during co-infection, many parameters may be altered, leading to increased susceptibility to co-infection: increased epithelial damage,

decreased mucociliary function, decreased production of antimicrobial peptides (AMPs), and impaired or enhanced cytokine and chemokine production.

One of the hallmarks of IAV infection is lung epithelial damage. This lung damage can be caused directly by the virus via the viral cytotoxic peptide PB1-F2 (McAuley *et al.* 2009), but can also be induced by the antiviral immune response. One such component of the antiviral response, type I IFNs, is associated with immunopathology and lung damage when produced in high amounts (Herold *et al.* 2008; Högner *et al.* 2013; Davidson *et al.* 2014). Type I IFNs induce DR5 on airway epithelia following IAV infection, and interaction with its corresponding ligand TRAIL from monocytes leads to apoptosis of the epithelium (Holoch and Griffith 2009; Benedict and Ware 2012; Davidson *et al.* 2014; Ellis *et al.* 2015) in order to eliminate virally infected cells. As a synergistic increase in type I IFNs following co-infection has been observed (Nakamura *et al.* 2011), this highlights the possibility that a synergistic increase in type I IFNs is contributing to the amplified lung damage observed within co-infected lungs. Another death receptor, FAS, which can be induced by type I IFN, can also bind its ligand FASL to induce apoptosis (Waring and Müllbacher 1999; Fujikura *et al.* 2013). It is not yet known how these death receptors are affected following co-infection, and whether the combination of the viral and bacterial stimuli results in increased expression on AECs.

This lung damage following IAV infection has been shown to increase mucus production and decrease ciliar beat frequency (McCullers and Rehg 2002). This severely impacts the mucociliary clearance function of the airway epithelium to trap and remove pathogens, and thus provides an ideal environment for bacterial binding, colonization and dissemination (Plotkowski *et al.* 1986; Pittet *et al.* 2010; Goulding *et al.* 2011).

AMPs can be produced by the epithelia to help control bacteria through direct bacterial killing or inhibiting binding (Bals 2000; Nisapakultorn *et al.* 2001; Wiesner and Vilcinskas 2010). How these are affected by the primary IAV infection, the antiviral immune response, or during co-infection is as of yet unknown.

The primary antiviral response can result in immune impairment. This can be mediated through the desensitization of TLR signalling in innate immune cells (Didierlaurent *et al.* 2008), and result in the reduced production of CCL2, required for the recruitment of monocytes (Nakamura *et al.* 2011), CXCL1 and CXCL2, required for the recruitment of neutrophils (Shahangian *et al.* 2009). This immune impairment may help reduce the possibility of over-activating the immune response that may lead to immunopathology resulting in increased lung damage. Unfortunately, this can contribute to bacterial escape from the immune response, resulting in outgrowth and bacterial-induced damage. Whether the epithelial production of chemokines in response to bacteria is impaired following primary infection remains to be elucidated.

Although there are reports of impaired antibacterial responses following IAV infection, a massive proinflammatory cytokine production has been documented within the lungs of co-infected mice, which correlates with mortality (LeVine *et al.* 2001; Seki *et al.* 2004; Damjanovic *et al.* 2013; Ellis *et al.* 2015). A high concentration of proinflammatory cytokines within the lung can be directly toxic, as they can induce cell death, and they can further drive inflammation, by recruiting and activating innate immune cells, all contributing to pathology in the lung. This increased damage could allow for enhanced bacterial outgrowth (Ellis *et al.* 2015) and dissemination that could ultimately lead to sepsis and death of the host. It is therefore possible that the epithelial response to the combined viral and bacterial

stimuli is greater than the response to single stimuli, resulting in an excessive proinflammatory cytokine production.

Overall, there are multiple factors contributing to bacterial susceptibility following influenza infection. It is clear that the host response to the viral and bacterial infection plays a pivotal role in the progression of the disease. Since the airway epithelium initiates and contributes to the host response to co-infection, epithelia may play a vital role in determining the outcome of disease. It is therefore important to study the epithelial reaction to sequential viral and bacterial stimuli and compare this to single stimuli.

3.2 Hypothesis and Aims

As the upper airway epithelial cells are direct targets of many respiratory pathogens, and communicate with the immune system, their responses may underlie the symptoms observed in influenza – *Streptococcus pneumoniae* co-infection, namely: lack of bacterial control, increased lung damage, and massive pulmonary inflammation. To understand the role of epithelia in each of these processes, primary airway epithelia were grown *in vitro* to study in isolation the epithelial responses to IAV – bacterial co-stimulus compared to responses to both single stimuli.

I hypothesize that:

Compared to single stimuli, co-stimulation of AECs will result in:

- A suppression of AMPs, which would allow for the outgrowth of bacteria
- Increased production of type I and III IFNs, resulting in increased ISG induction
- Upregulation of apoptosis related genes, contributing to increased lung epithelial cell death
- Massive proinflammatory cytokine and chemokine induction

3.3 Results

3.3.1 Selection of infection stimuli

The main inducer of the effects observed during co-infection *in vivo* has not yet been identified. Given that airway epithelial cells (AECs) are the first host target of both IAV and *S. pneumoniae*, they may be an important driver of these effects. Primary mouse tracheal epithelial cell (mTECs) cultures are a useful experimental system of isolating AECs from the rest of the immune system to determine their responses through the induction of AMPs, IFNs, ISGs, apoptosis inducing ligands and proinflammatory cytokines and chemokines. mTECs were derived from C57BL/6 mice, given that the majority of the literature utilise this in-bred strain, allowing for direct comparisons. Many studies on co-infection *in vivo* infect with A/Puerto Rico/8/1934 (H1N1) (PR8) (Bouvier and Lowen 2010). PR8 induces severe lung damage, morbidity and mortality in mice unlike many other human isolates of influenza. Therefore, in order to study the effect of co-infection on the epithelia without a severe initial viral infection, a milder influenza strain was chosen – X31. X31 is a re-assortment of the PR8 strain with the HA and NA of A/Hong Kong/1/1968 (H3N2) (Tam *et al.* 2013). This strain induces only moderate disease allowing for assessment of synergy with the secondary bacterial infection.

The common laboratory strain of *Streptococcus pneumoniae,* D39*,* is used in many mouse models of infection (Chiavolini *et al.* 2008) and in co-infections (Goulding *et al.* 2011). It is a mild strain of *S. pneumoniae,* where its virulence depends on the strain of mice, route of administration and dosage administrated. Live D39, and heat-killed (HK) D39 which would act as a bacterial stimulus without the ability to proliferate and damage the epithelia, or overgrow in tissue culture, were used. In lieu of the bacterium and as a clean positive control, Toll-like

receptor agonists were also used - Pam3CSK4, a TLR2 agonist, and lipopolysaccharide (LPS), a TLR4 agonist. The virus was placed on the cells for 24 hours, then the bacterial stimulus was added for a further 24 hours. A synergistic effect of a co-infection must be greater than the sum of the induction from the virus and the bacterial stimulation alone.

To assess whether AECs respond to viral and bacterial stimulation, the known virus-induced type III IFN, IFNλ (IL-28A), and the known bacterial- and virus-induced CXCL2 were analysed (Figure 5). The virus induced IL-28A as expected, whereas the bacterial agonist, Pam3CSK4, did not (Figure 5A), which confirms these AEC cultures can respond to virus infection. Pam3CSK4 did however upregulate CXCL2 as expected, along with the virus, confirming the epithelia can also respond to bacterial stimulation (Figure 5B).

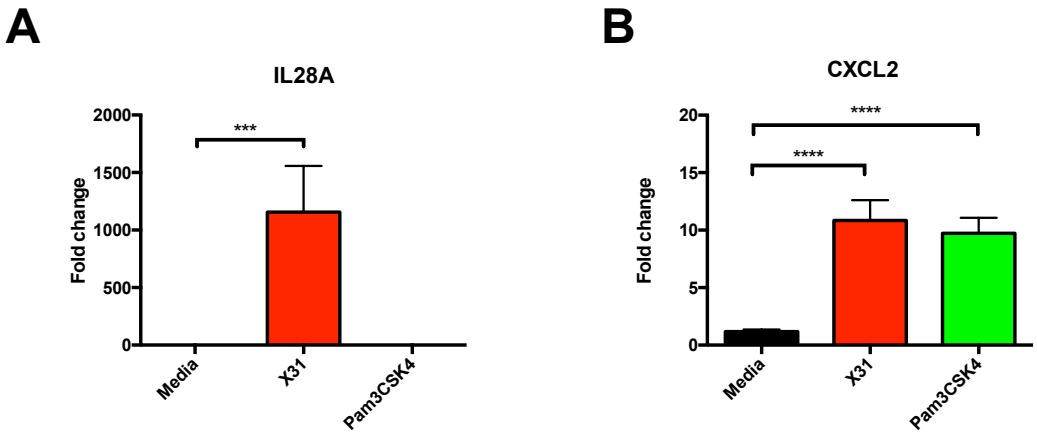


Figure 5 AECs respond accordingly to viral and bacterial stimulation

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 or exposed to Pam3CSK4 (1 µg/ml) for 24 hours. cDNA from the cultures were analysed for IL28A induction and CXCL2 induction. Significance was assessed by unpaired t-test where *** P<0.001, **** P<0.0001. Graphs show mean ± SEM. Data pooled from four independent experiments, n=6-24.

3.3.2 AMP production by AECs is minimally induced by bacteria

AMPs produced by AECs can directly kill invading bacteria, and lack thereof may allow for increased colonisation of the lung. How their induction is affected during co-exposure is unknown. We hypothesise that preceding viral infection suppresses AMP production, thus contributing to lack of bacterial control in co-infection. Six antimicrobial peptides were chosen: Defb1, Defb2, Defb3, CAMP, Reg3g and S100a8, to asses their induction and possible suppression following co-infection. It is surprising to see that bacterial stimulation alone does not induce any of these AMPs significantly higher than the media control (Figure 6). There is a statistically significant induction of Defb1 when X31 is combined with LPS (1 µg/ml) compared to either single stimulus. Although not statistically significant, expression of Defb1 increases when the lower concentration of LPS is used following IAV infection, and with both concentrations of Pam3CSK4. Defb3 displays a marginal increase following X31 + HK *S. pneumoniae,* and an increase in Reg3g when X31 is combined with Pam3CSK4 (1 µg/ml), LPS, or live *S. pneumoniae*. These increases are in contrast to the suppression that was hypothesized. Since bacterial stimulation alone does not seem to induce antimicrobial peptides, their subsequent suppression following co-exposure is therefore difficult to determine. In addition, a statistically significant increased induction in the presence of combined stimuli was observed in one case, which is difficult to reconcile with *in vivo* observations of reduced bacterial control.

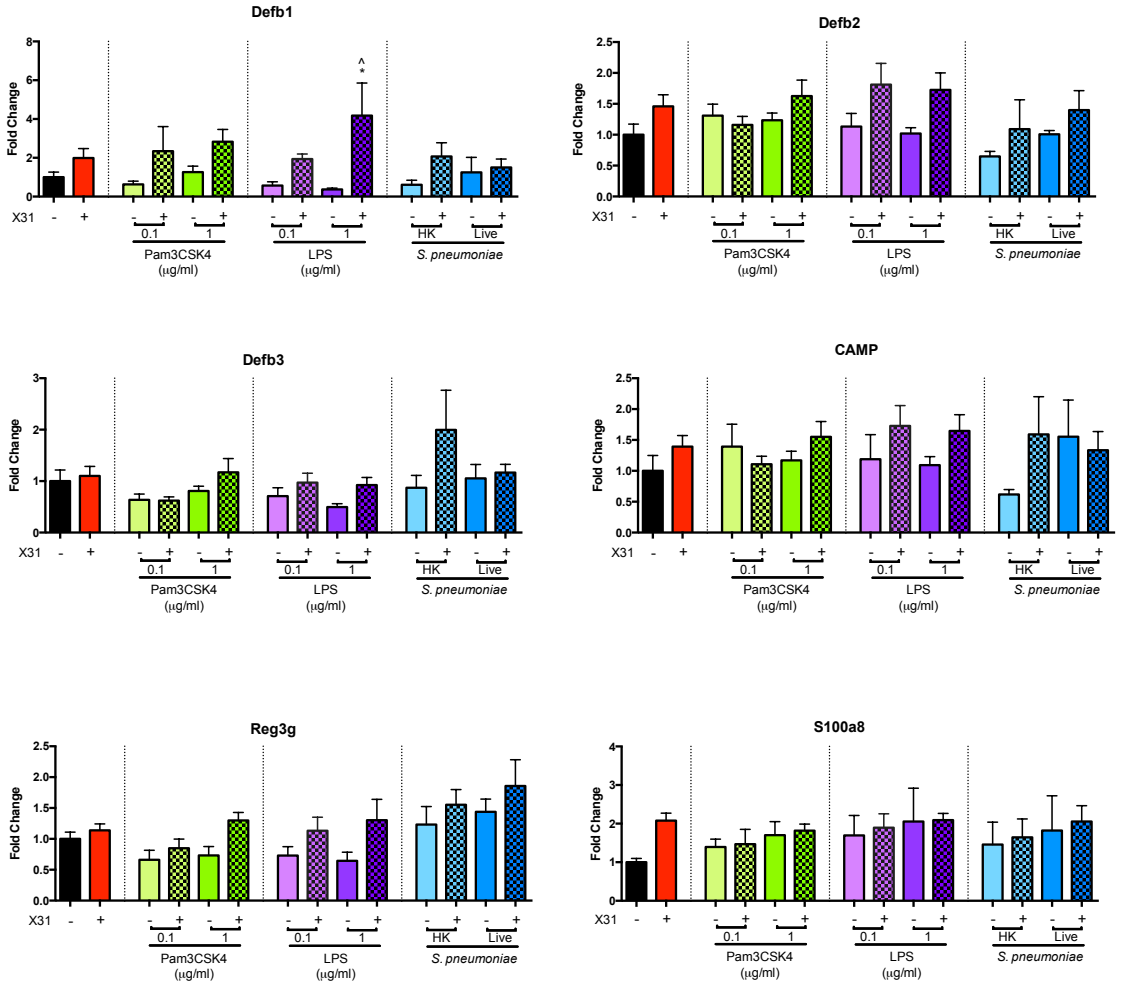


Figure 6 The induction of AMPs by bacterial stimuli is minimal and generally remains unmodified by viral co-stimulus

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then stimulated with either Pam3CSK4, LPS, HK or live *S. pneumoniae* (10^6 CFU/well) for a further 24 hours. cDNA from the cultures were analysed for antimicrobial peptides. Significance was assessed by unpaired t-test where * indicates co-stimulus:single bacterial stimulus, ^ indicates co-stimulus:X31. * $P<0.05$. Significance only displayed if co-stimulus is greater than each single stimulus. Graphs show mean ± SEM. Data pooled from four independent experiments, n=3-16.

3.3.3 IAV infection dominates the induction of type I and III IFNs, and subsequently ISG induction

Since type I IFN production has been associated with many immune impairment and pathogenic effects, type I and III IFNs were quantified. Viral infection of AECs induces the production of IFNα, IFNβ and IFNλ. However, bacterial stimulation alone rarely induces any IFNs (Figure 7). IFNa4 messenger levels never exceed the media control for the virus or bacterial stimulants (Figure 7A). There is an induction of IFN protein by the viral infected epithelia as expected, and by both heat killed and live *S. pneumoniae* treatments, however this is not significant (Figure 7B). Messenger levels of IFNb1 and IL28A (IFNλ) can be observed by X31 and live *S. pneumoniae* treatment, however protein is detected only following X31 infection.

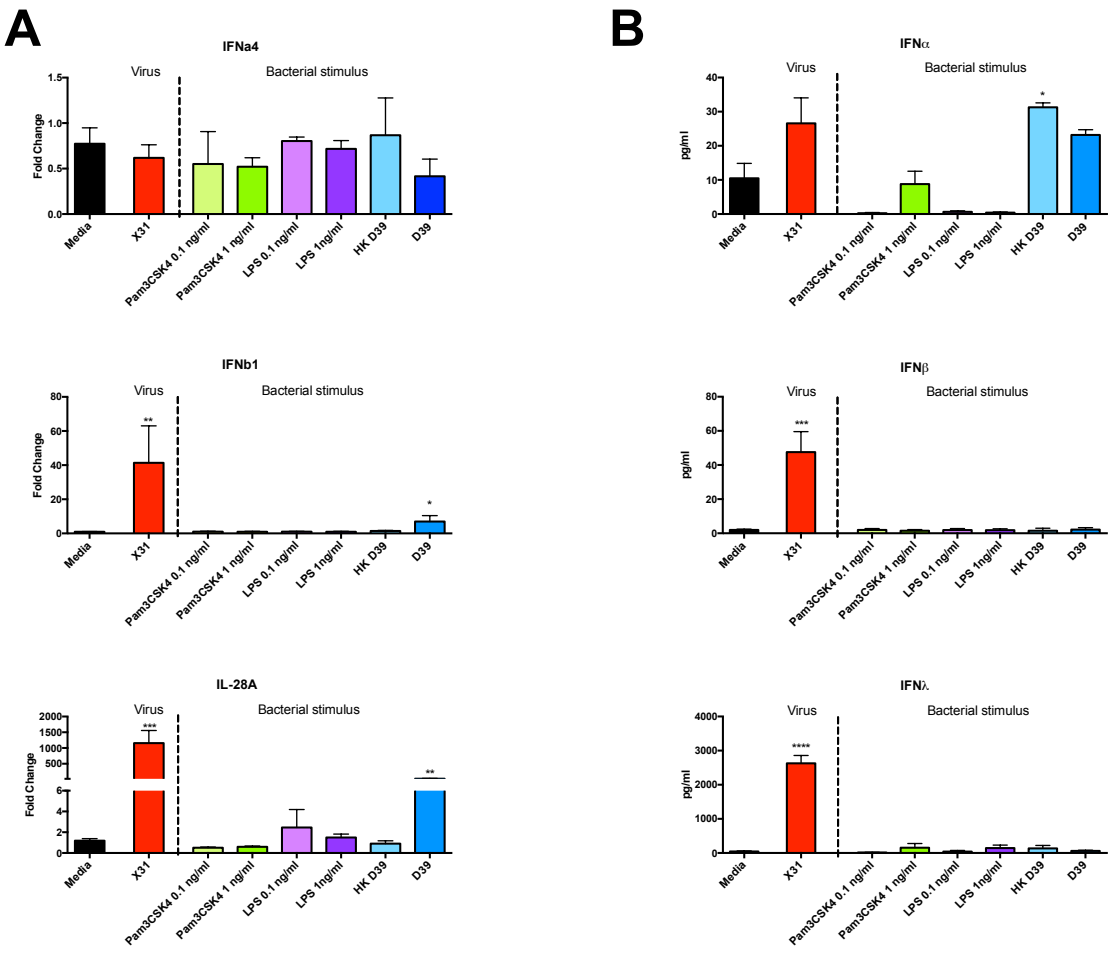


Figure 7 Viral but not bacterial stimulation of AECs induces an IFN response

C57BL/6 epithelia were treated with X31 (MOI: 0.1), Pam3CSK4, LPS, HK or live *S. pneumoniae* (D39, 10^6 CFU/well) for 24 hours. cDNA from the cultures were analysed for IFNa4, IFNb1 and IL28A (A). Supernatants were collected and analysed for IFNα, IFNβ, and IFNλ (B) Significance compared to media control and assessed by unpaired t-test where * P<0.05, ** P<0.01, *** P<0.001. Graphs show mean ± SEM. Data pooled from six independent experiments, n=3-24.

When the two stimuli are joined there is no great synergy observed. Only messenger levels of IFNa4 when X31 and live *S. pneumoniae* are combined, and both messenger and protein levels of IFNλ when X31 and 0.1 μg/ml of LPS are combined, result in a statistically significant increase compared to the single stimuli (Figure 8). IFNa4 messenger levels, although not statistically significant shows an increase when IAV is combined with HK *S. pneumoniae* (Figure 8A), and in IFNα protein levels when X31 is combined with HK and live *S. pneumoniae* (Figure 8B). There is an increase in IFNβ protein levels following IAV, and HK or live *S. pneumoniae* combination (Figure 8B). An increase in IFNλ protein levels can also be observed when the virus is combined with Pam3CS4, LPS, HK or live *S. pneumoniae* (Figure 8B). However, the majority of these increases are not statistically significant, therefore it is predominantly the antiviral response that drives the induction of type I and III IFNs. This is not surprising in the light of Figure 7 where the bacteria alone rarely induced IFNs to begin with, but it is in disagreement with *in vivo* findings by Nakamara *et al.* (Nakamura *et al.* 2011). This may be due to the altered order of administration as the bacteria were given before IAV within this study.

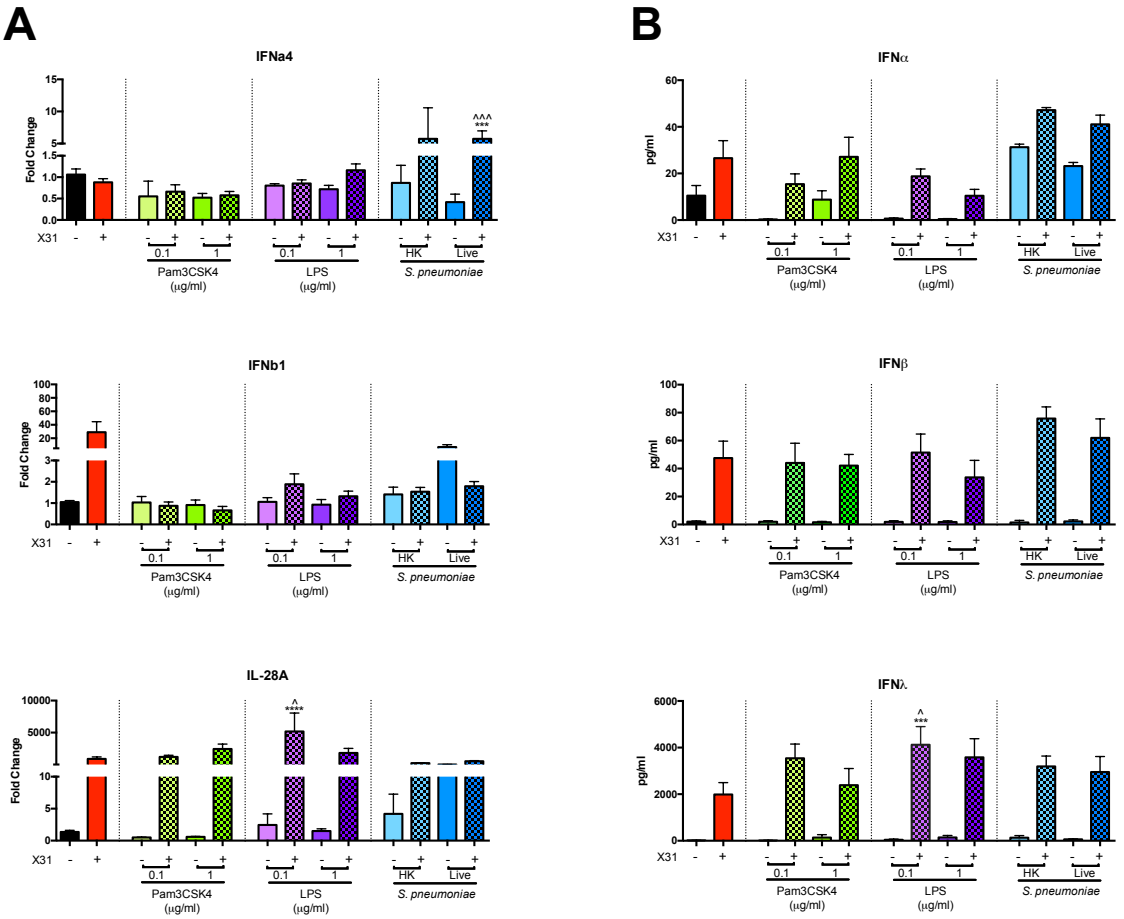


Figure 8 A viral-bacterial co-stimulation of AECs results in only marginal increases in the production of IFNs

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then with either Pam3CSK4, LPS, Heat killed (HK) or live *S. pneumoniae* (D39, 10^6 CFU/well) for a further 24 hours. cDNA from the cultures were analysed for IFNa4, IFNb1 and IL-28A (A). Supernatants were collected and analysed for IFNα, IFNβ and IFNλ (B) Significance was assessed by unpaired t-test where * indicates co-stimulus:single bacterial stimulus, ^ indicates co-stimulus:X31. * P<0.05, *** P<0.001, **** P<0.0001. Significance only displayed if co-stimulus is greater than each single stimulus. Graphs show mean ± SEM. Data pooled from six independent experiments, n=3-24.

Given the lack of type I or type III IFN induction by the bacterial stimulus alone and only marginal increases following a co-stimulus, it is therefore not surprising to see little to no induction of ISGs by the bacterial stimulation alone (Figure 9). No significant synergy or suppression is observed when the virus is combined with the bacterial stimuli. There appears to be a non-significant suppression of Rsad2 and Oasl2 when the virus is combined with HK or live *S. pneumoniae*. This suppression also appears for ifi203 when X31 is combined with both concentrations of Pam3CSK4 and LPS, but again this is not significant. There is however an increase in Oasl2 following X31 + Pam3CSK4 and X31 + LPS stimulation, but yet again these are not significant (Figure 9). Overall, the levels of induction are in general similar to the inductions by the virus alone, which further confirms that viral infection dominates the epithelial response thus far.

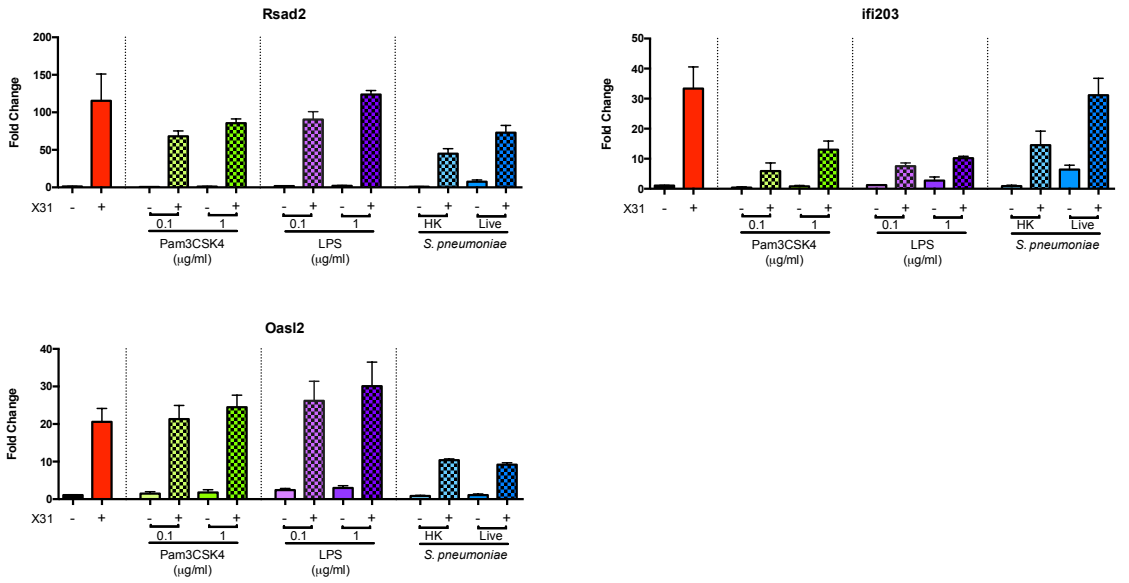


Figure 9 IAV infection of AECs induces ISGs, which are unchanged following a co-stimulation

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then with either Pam3CSK4, LPS, HK or live *S. pneumoniae* (D39, 10^6 CFU/well for a further 24 hours. cDNA from the cultures were analysed for interferon stimulated genes: Rsad2, ifi203 and Oasl2. No significance for any co-stimulation:single stimulation. Graphs show mean ± SEM. Data pooled from five independent experiments, n=3-24.

3.3.4 Death receptors are not induced in isolated mTEC cultures

Given the data of lung damage facilitating bacterial colonisation and pathology in co-infected lungs (McAuley *et al.* 2009; Ellis *et al.* 2015), apoptosis-related genes were quantified. Lung damage observed *in vivo* correlated with an increase in DR5 on AECs (Ellis *et al.* 2015). DR5 can be induced on airway epithelia by type I IFNs, an antiviral cytokine, which can bind to its receptor TRAIL, another ISG, to induce apoptosis of the DR5 expressing cell. However, upon testing DR5 messenger levels on AECs *in vitro*, it was found that the virus alone did not induce it, nor do bacterial stimulation alone, or the combination (Figure 10). Viral and bacterial stimulation induces TRAIL, which is significantly increased following co-stimulation of IAV with Pam3CSK4 or LPS, compared to both single stimuli. There is no statistically significant increase over single stimuli when either the HK or live *S. pneumoniae* are used. HK and live *S. pneumoniae* marginally increase expression of FAS, another death receptor, however this is not significant. Following X31 and live *S. pneumoniae* co-stimulation there appears to be a suppression of FAS, but this is also not significant. Viral stimulation alone drives the induction of its ligand FASL, whereas bacterial stimulation alone does not. There is a significantly increased induction of FASL when the virus and the higher concentration of LPS are combined in comparison to the two single stimuli. Overall, the apoptosis-related receptors are not induced. This may indicate the type I IFN amounts produced within these cultures are not sufficient to induce these death receptors, or that other cytokines or cell interactions, not present in our mTEC system, are required on top of IFNs to upregulate death receptors on epithelia. However, the epithelia can significantly increase the ligands following co-infection compared to single stimuli,

which suggests that epithelial cells may contribute to the induction of apoptosis of the epithelium when the death receptors are present.

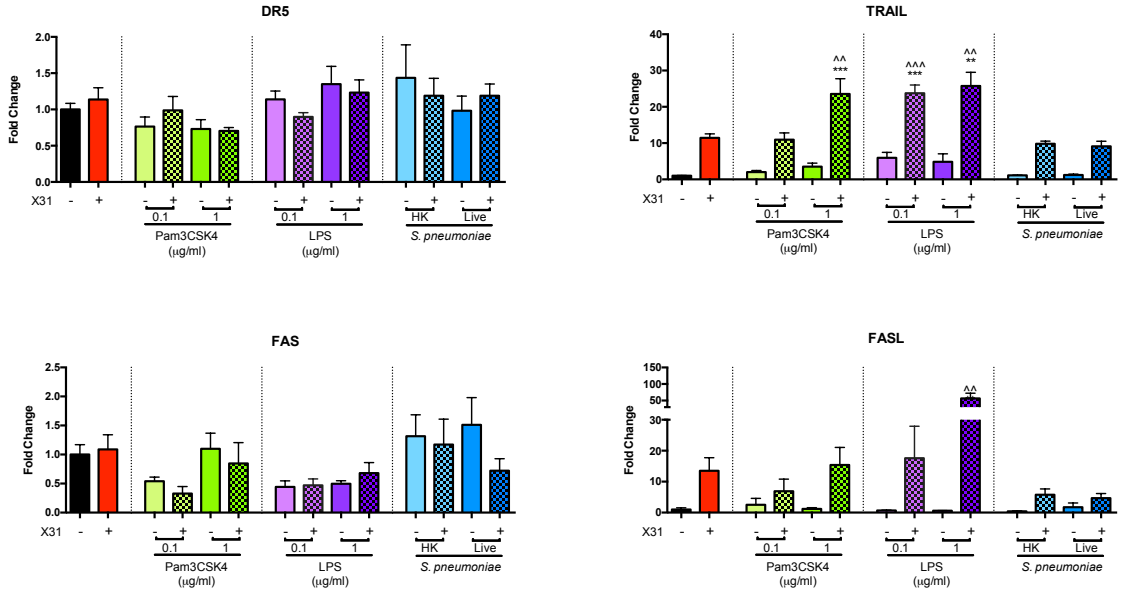


Figure 10 Co-stimulation of AECs does not induce death receptors more strongly than in the corresponding single infections

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then with either Pam3CSK4, LPS, HK or live *S. pneumoniae* (D39, 10^6 CFU/well) for a further 24 hours. cDNA from the cultures were analysed for apoptosis related genes, Death receptors: DR5 and FAS with their corresponding ligands: TRAIL and FASL respectively. Significance was assessed by unpaired t-test where * indicates co-stimulus:single bacterial stimulus, ^ indicates co-stimulus:X31. ** P<0.01, *** P<0.001. Significance only displayed if co-stimulus is greater than either single stimulus. Graphs show mean ± SEM.

3.3.5 Co-stimulation of AECs results in increased induction of proinflammatory cytokines and chemokines

Cytokines and chemokines can be protective during infections, but excessive amounts can be pathogenic and therefore detrimental to the lung. Co-stimulation of AECs induces statistically significant increases in messenger and protein levels of the proinflammatory cytokines CXCL1, CXCL2 and TNF-α when Pam3CSK4 and LPS are used as the bacterial stimulus (Figure 11). Combination of the virus and HK *S. pneumoniae* also induces a significant increase in messenger levels of CXCL2, however this appears to be an additive effect (Figure 11A). An additive effect, where the sum of the two stimuli alone equals the induction by the combination, also appears to be responsible for the increase in messenger IL-6 levels when X31 and live *S. pneumoniae* are combined (Figure 11A). Protein levels of IL-6 however display a significant increase when X31 is combined with the lower concentration of Pam3CSK4, and with both concentrations of LPS. An additive induction of IL-6 can be observed following the combination of virus and the higher concentration of Pam3CSK4, but this is not significant in comparison to single stimuli (Figure 11B). Interestingly, there appears to be a reduction in IL-6 protein, which does not correlate with a decrease in messenger levels following viral and live *S. pneumoniae* stimulation. There is a significant increase in protein levels of TNF-α following X31 and live *S. pneumoniae* co-stimulation. However, this is not as great as when LPS is used as the secondary stimulus (Figure 11B). Overall, co-exposure of epithelia leads to a synergistic increase in a range of proinflammatory cytokines and chemokines. This indicates that the airway epithelia are contributing to the recruitment of neutrophils and other innate immune cells crucial for controlling and eliminating the bacteria. Virus-dependent suppression of these

chemoattractants, as has been described in the literature for other cell types, was not observed in mTECs.

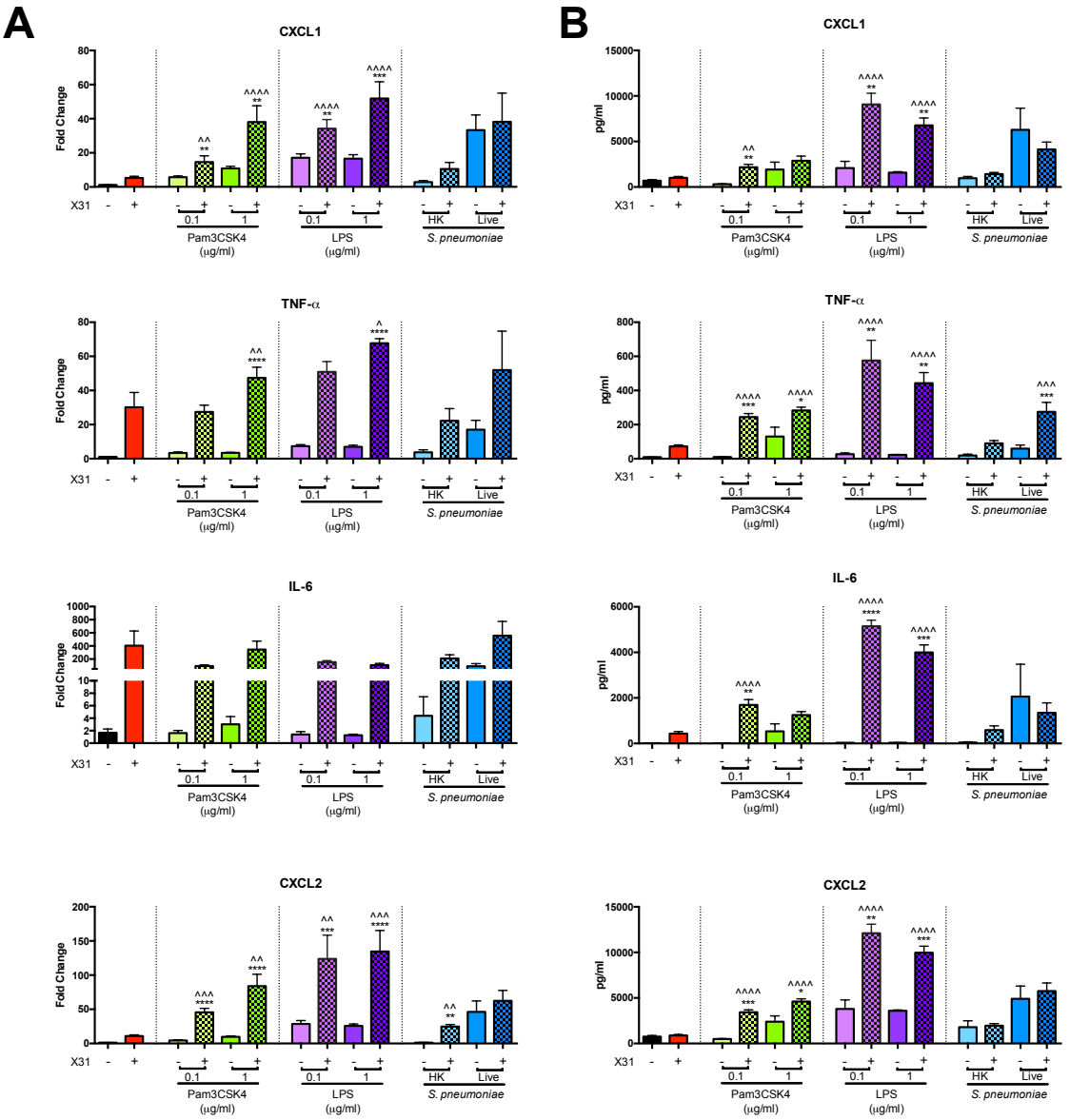


Figure 11 Co-stimulation with bacterial agonists on AECs results in greater proinflammatory cytokine induction compared to the two single stimuli.

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then with Pam3CSK4, LPS, HK or live *S. pneumoniae* (D39, 10^6 CFU/well) for a further 24 hours. cDNA from the cultures were analysed for CXCL1, TNF-α, IL-6 and CXCL2 (A). Supernatants were collected and analysed for CXCL1, TNF-α, IL-6 and CXCL2 ELISA and cytokine multiplex (B). Significance was assessed by unpaired t-test where * indicates co-stimulus:single bacterial stimulus, ^ indicates co-stimulus:X31. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Significance only displayed if co-stimulus is greater than either single stimulus. Graphs show mean ± SEM. Data pooled from seven independent experiments, n=3-40.

Using a hypothesis driven approach to analyse the cytokine and chemokine response may not be giving an overall view of the synergistic increases in cytokine levels. Therefore, a cytokine multiplex was performed. The fold changes of cytokines induced by co-stimulus over the corresponding single stimulus were calculated and graphed (Figure 12 and 14). Considering the TLR agonists as the secondary stimulus, it is clear to see that there is a greater fold induction of cytokines by the virus than the bacteria (Figure 12A, C). We took the top six most upregulated, and any downregulated cytokines and combined them into a Venn diagram (Figure 12B, D). In general we see that bacterial stimulation upregulates known bacterially induced cytokines such as CXCL2, and G-CSF. The virus is also upregulating known cytokines such as CXCL10 and CCL5. Although we find very little overlap, it is interesting to see that TNF-α is induced more strongly by co-stimulation with either Pam3CSK4 or LPS than by either single stimulus. CCL3 is also commonly suppressed by co-exposure compared to exposure to either single stimulus (Figure 12B, D). Furthermore, CXCL1 and IL-6 is more greatly induced by co-stimulation with LPS than either single stimuli (Figure 12D).

To ensure that the fold increases reported in Figure 12 are not simply due to very small cytokine amounts skewing the ratios, and to calculate statistical significance, we also looked at the absolute protein levels of the cytokines induced. Of the cytokines that overlap, TNF-α, and CXCL1 show the most striking and statistically significant increases by X31 and LPS co-stimulation over single stimuli (Figure 13A). IL-6 is also significantly increased although not as striking as with TNF-α and CXCL1 (Figure 13A). These increases also confirm the findings in Figure 12 obtained by individual ELISAs. Looking at the protein levels of the other most induced cytokines we find that for CXCL10 and G-CSF there are no

significant increases following co-exposure is observed (Figure 13B). However, there are significant increases in CCL2 and CXCL2 protein levels following co-exposure in comparison to both single stimuli, also confirming results from Figure 12. The suppression of CCL3 in co-stimulation was not significant versus either single stimulus (Figure 13C)

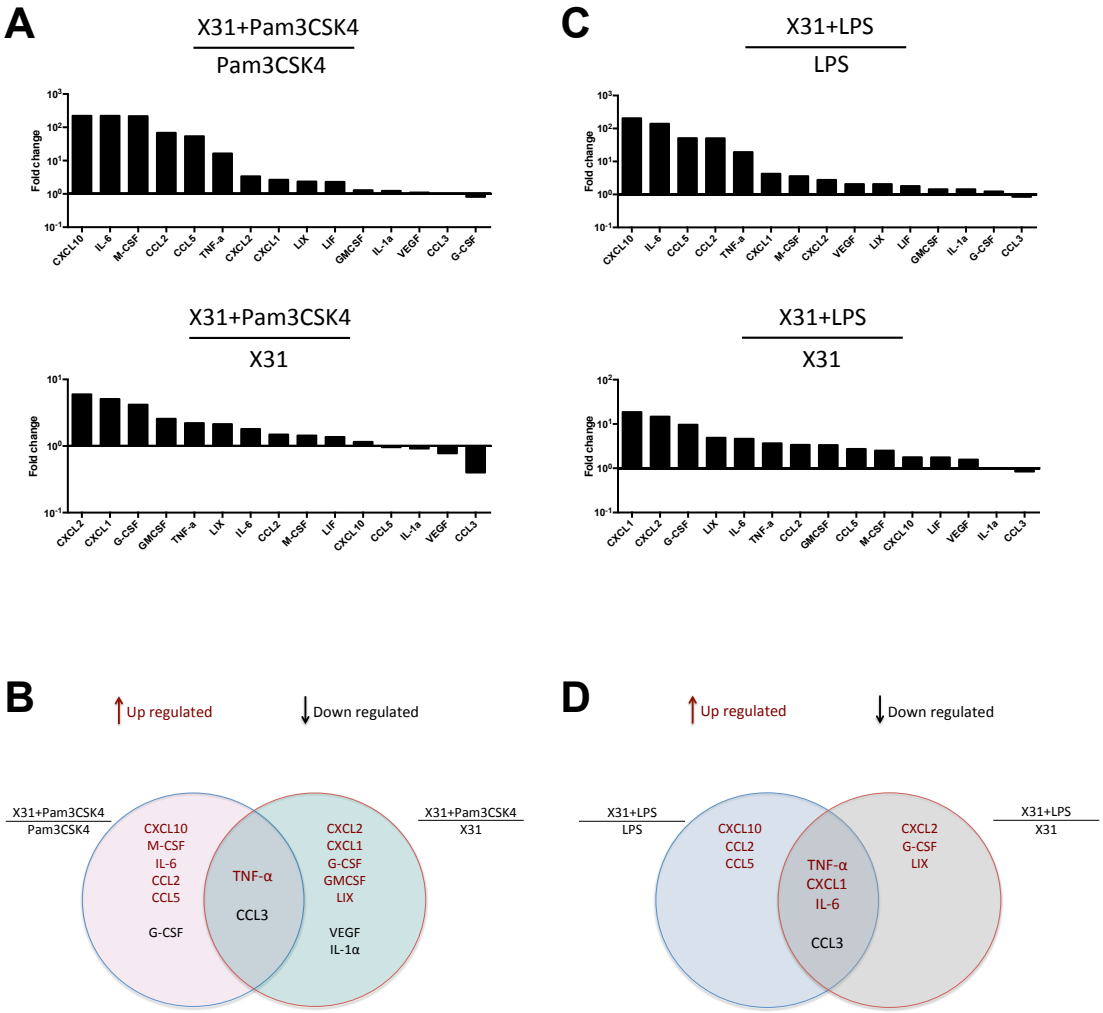


Figure 12 Only a small subset of cytokines are consistently induced or suppressed following X31 and TLR agonist co-exposure

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then exposed to either Pam3CSK4 or LPS (1 μg/ml) for a further 24 hours. Supernatants from the cultures were analysed by cytokine multiplex. Co-stimulation induced cytokine amounts were calculated as fold changes over the corresponding single stimulus (A, C). The six most upregulated, and cytokines that were downregulated were put into a Venn diagram for when Pam3CSK4 (B), or LPS (D) is used. Graphs show mean ± SEM. Data representative of 2 independent experiments, n=3-4.

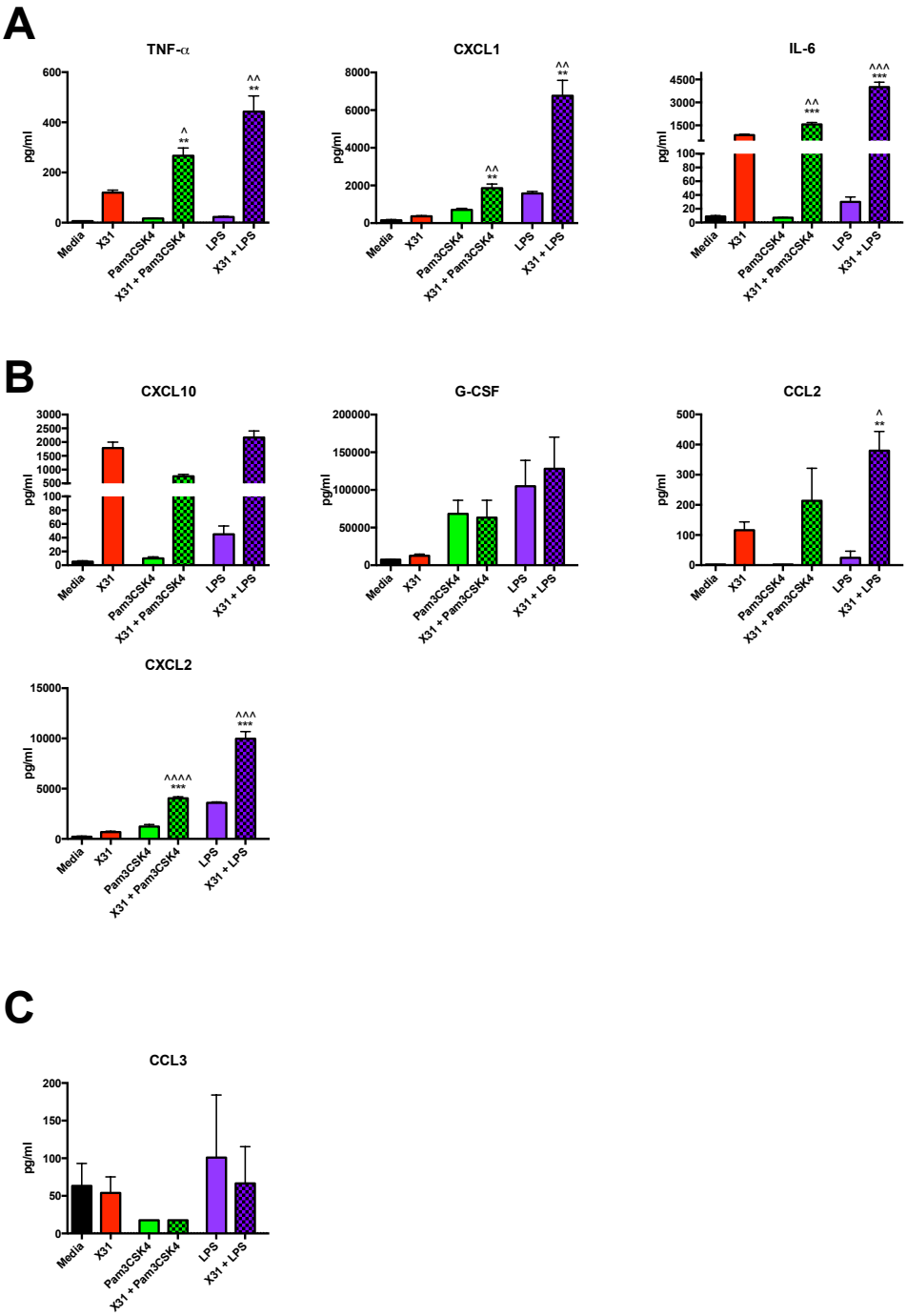


Figure 13 Absolute values show significant increases following co-exposure

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then exposed to either Pam3CSK4 or LPS (1 µg/ml) for a further 24 hours. Supernatants from the cultures were analysed by cytokine multiplex. Absolute values of the most upregulated overlapping cytokines (A), a subset of cytokines differentially regulated (B), and the overlapping downregulated cytokine (C) in Figure 12B and D. Significance was assessed by unpaired t-test where * indicates co-stimulus:single bacterial stimulus, ^ indicates co-stimulus:X31. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Significance only displayed if co-stimulus is greater than either single stimulus. Data representative of 2 independent experiments, n=3-4.

When *S. pneumoniae* is used as the secondary stimulus we again see a greater increase in cytokine fold changes by the virus than by either the HK or live *S. pneumoniae* (Figure 14A, C). This confirms that the virus represents a stronger stimulus for the epithelium than either the bacteria or bacterial stimulus do. It is interesting to note that the *S. pneumoniae* infection induces a different set of top cytokines compared to PAM3CSK4 or LPS. However we still detect induction of common antiviral cytokines, CXCL10 and IFNγ, and common antibacterial cytokines, CXCL1, and G-CSF (Figure 14B, D). Interestingly, CCL5 is commonly up regulated by co-stimulation with IAV and either HK or live *S. pneumoniae* in comparison to the single stimuli (Figure 14B, D). TNF-α is also most up regulated when live *S. pneumoniae* is used as the secondary stimulus as was expected based on the results obtained with bacterial TLR agonists in Figure 12 (Figure 14B). IL-6 on the other hand is more upregulated by IAV co-exposure with HK *S. pneumoniae* (Figure 14D). VEGF is commonly down regulated during X31 and live *S. pneumoniae* co-stimulation (Figure 14B), which was also down regulated by Pam3CSK4 (Figure 14B).

Assessing absolute protein levels we find that apart from TNF-α, there is no significant increase by co-stimulation in comparison to the two single stimuli for CCL5 and IL-6. IL-6 again appears to be suppressed when X31 is combined with live *S. pneumoniae*. TNF-α is significantly increased by X31 and live *S. pneumoniae* co-exposure (Figure 15A). A subset of the top induced cytokines (Figure 15B) and those downregulated (Figure 15C) in Figure 14 also do not show any significant increase by co-exposure in comparison to both single stimuli, apart from G-CSF when X31 is combined with live *S. pneumoniae.*

The cytokines are not filtered on statistical significance before generating fold changes. This is why some cytokines come out as highly induced yet are not significantly changed in protein levels. Overall, Figures 12-15 show epithelia respond separately to viral and bacterial stimulation and the cytokine profiles are relatively separated, with those cytokines that are induced mainly by the viral infection and those which are induced mainly by bacterial stimulation. There are a few exceptions of cytokines that are synergistically induced by co-exposure: TNF-α by virus plus TLR agonists or bacteria; IL-6, CXCL2, CCL2 and CXCL1 only by virus plus TLR agonists, and CCL5 only by virus plus bacterial exposure. As these synergistically induced cytokines and chemokines have great proinflammatory potential, it would be interesting to test whether increased amounts of these cytokines contribute to immunopathology *in vivo*.

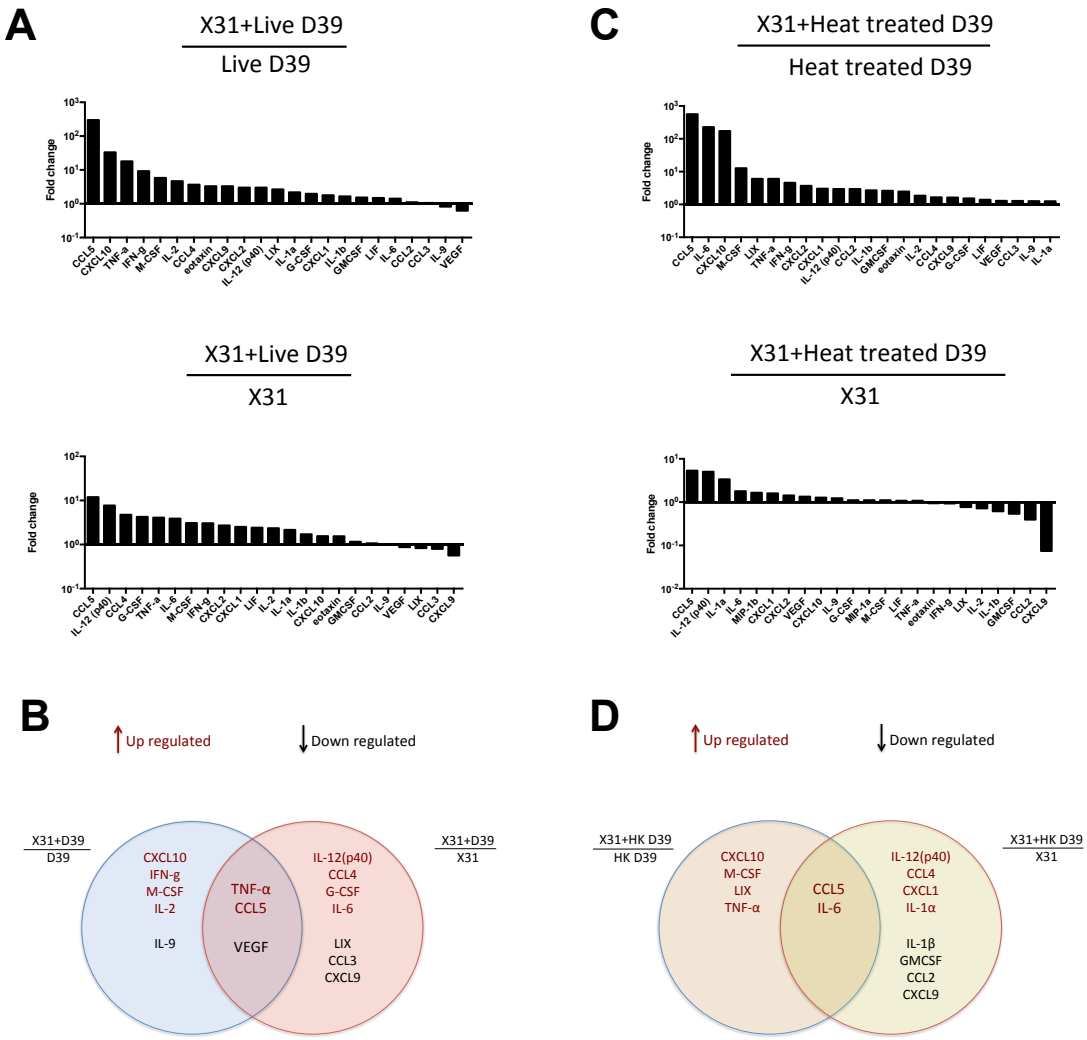


Figure 14 Only a small subset of cytokines overlap following X31 and *S. pneumoniae* co-exposure

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then with either HK or live *S. pneumoniae* (D39, 10^6 CFU/well) for a further 24 hours. Supernatants from the cultures were analysed by cytokine multiplex. Co-infected results were calculated as fold changes over the corresponding single infection (A, C). The six most upregulated, and cytokines that were downregulated were put into a Venn diagram for when live D39 (B), or HK D39 is used (D). Graphs show mean ± SEM. Data is representative of four independent experiments, n=3-4.

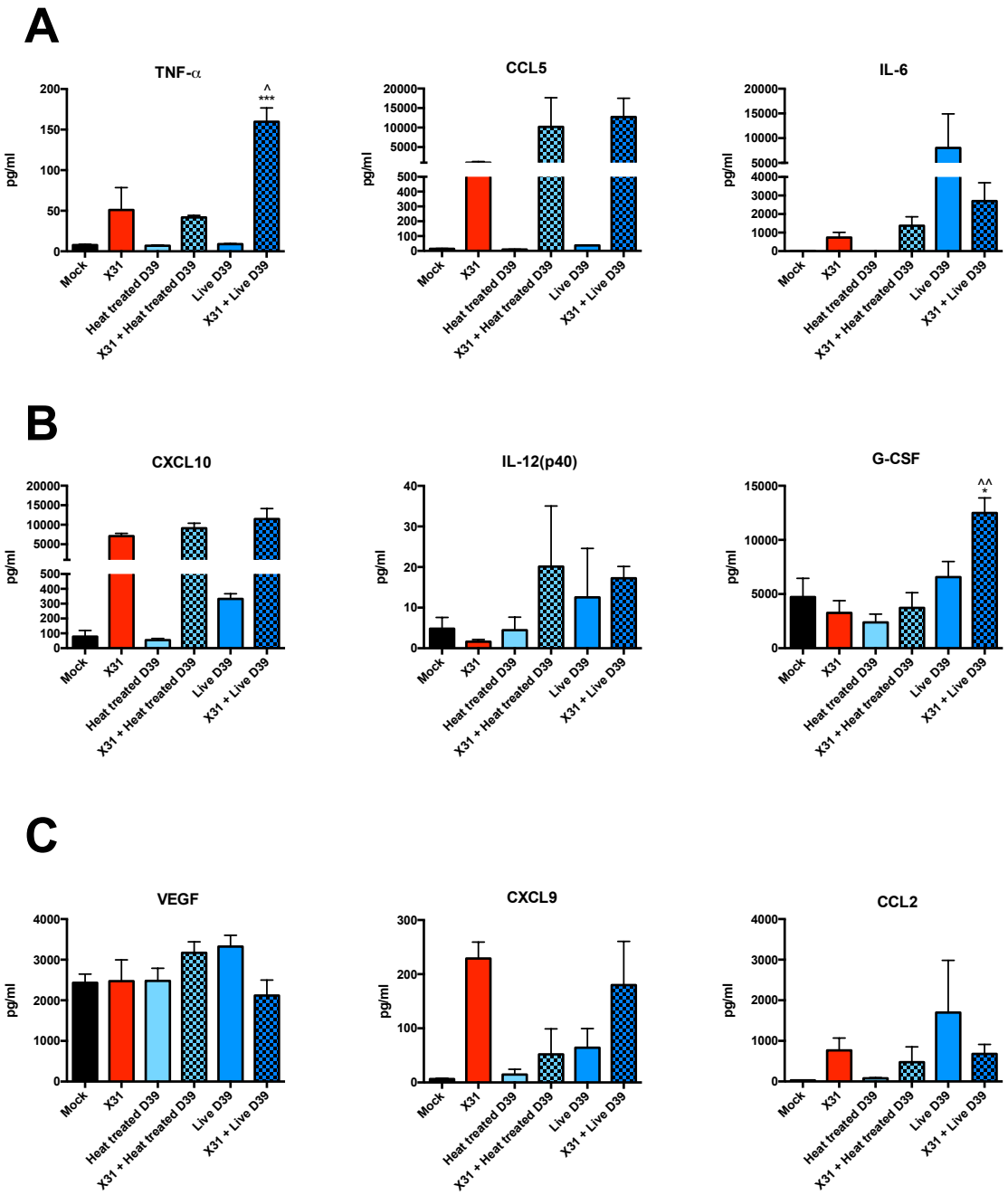


Figure 15 Absolute values for cytokines differentially regulated following IAV and *S. pneumoniae* co-exposure

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then with either HK or live *S. pneumoniae* (D39, 10^6 CFU/well) for a further 24 hours. Supernatants from the cultures were analysed by cytokine multiplex. Absolute values of the most upregulated overlapping cytokines (A), a subset of cytokines differentially regulated (B) and downregulated (C) in Figure 14B and D. Significance was assessed by unpaired t-test where * indicates co-stimulus:single bacterial stimulus, ^ indicates co-stimulus:X31. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Significance only displayed if co-stimulus is greater than either single stimulus. Data representative of 2 independent experiments, n=3-4.

3.3.6 IAV infection dominates the transcriptional response of the AECs

Expression profiling allows a global analysis of the transcriptional response of epithelia to exposure to virus or bacteria or both. We therefore performed microarray analysis on media control AECs, and AECs infected with X31 +/- LPS, Pam3CSK4, or live *S. pneumoniae*. Samples were normalized to the average of the media control group and filtered for a fold change of 2 ($p<0.01$) yielding 2731 genes differentially expressed in the experiment utilising LPS as the secondary stimulus (Figure 16A), and 1095 genes when live *S. pneumoniae* was used as the secondary stimulus (Figure 17A).

Concentrating on when LPS was used as the secondary bacterial stimulus, it is clear to see that the antiviral response dominates the overall transcriptional response (Figure 16A). This gene set was further filtered for a 2-fold change of X31 + LPS stimulation over X31 stimulation alone (Figure 16B) and X31 + LPS compared to LPS stimulation alone (Figure 16C). Co-stimulation leads to an additional 567 differentially expressed genes compared to virus stimulus alone, which is far fewer than the 2240 genes induced by co-stimulation over bacterial stimulus alone, which is a consequence of the stronger gene induction by virus exposure. A set of 505 genes were further upregulated upon costimulation as compared to both the virus and bacterial single stimulus (Figure 16D, E. Appendix Table 4). Genes of note that are differentially expressed are the cytokines: CXCL1, CXCL2, IL-6, IL-1α, TNF; the antimicrobial peptides: S100a9, S100a8, Slpi, and the apoptosis related gene: Tnfrsf19 (Appendix, Table 4). This list of 505 genes is represented as a heatmap (Figure 16E) and was used for Ingenuity Pathway Analysis (IPA) where the top 6 canonical pathways are represented (Figure 16F). It is unsurprising that the highest pathway is related to LPS signalling since LPS is

the secondary stimulus here, and that granulocyte recruitment processes are found, given the chemoattractants we have detected. It is also interesting that aryl hydrocarbon receptor related pathways appear twice among the top six pathways.

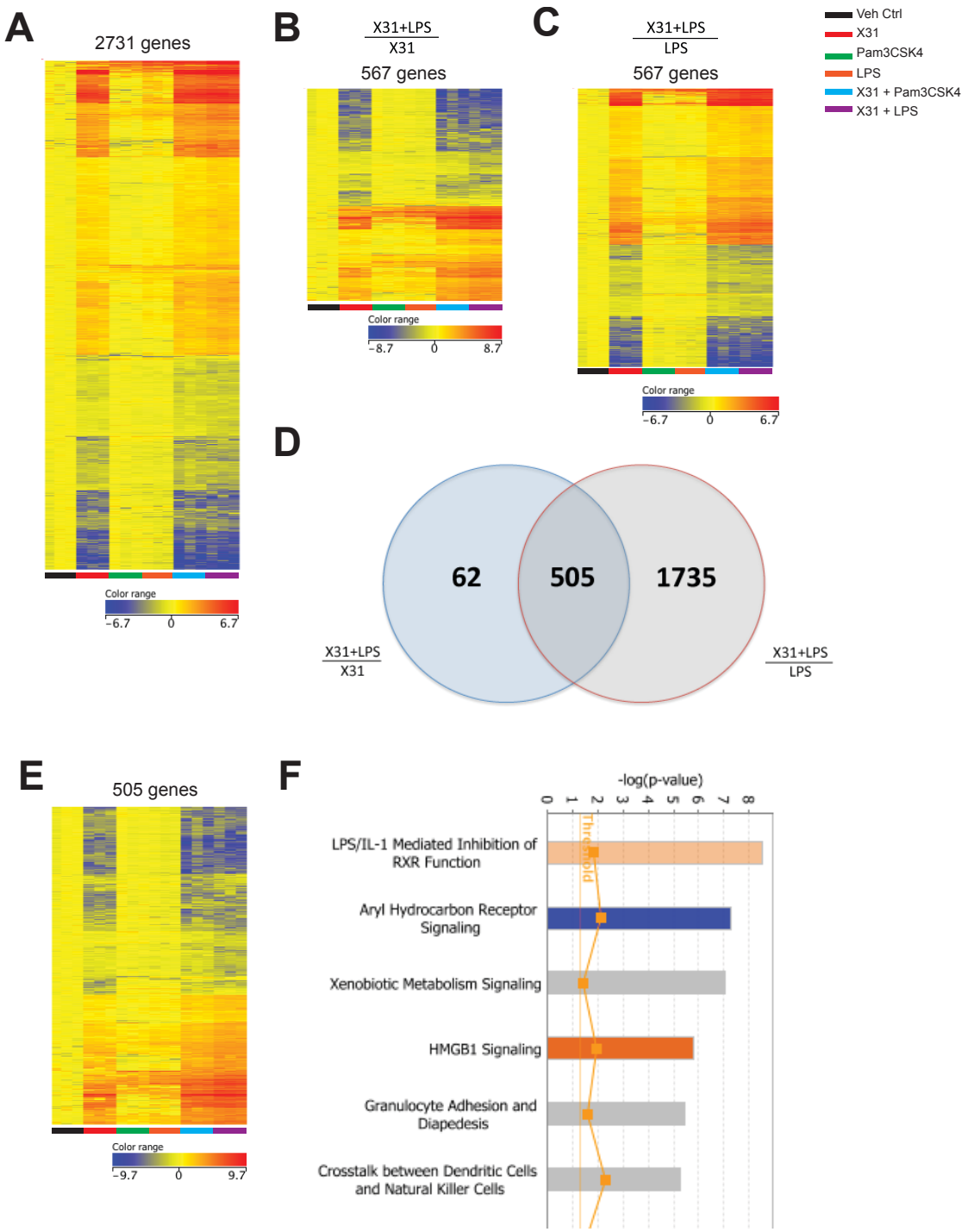


Figure 16 The antiviral response dominates the overall transcriptional response to co-stimulation with TLR agonists

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours, then exposed to either Pam3CSK4 or LPS for a further 24 hours. RNA was collected for global analysis by Illumina.SingleColor.Mouse WG-6_V2_0_R0_1127 microarrays. Samples (n=3) were normalized to the median of the vehicle control group and filtered for a fold change of 2 (A). This was subsequently filtered for 2-fold change of co-stimulation with LPS versus X31 (B), or versus

LPS alone (C). A Venn diagram was generated using the gene set from B and C to generate a list of genes differentially expressed in co-stimulation versus either single stimulation (D). The 505 genes commonly differentially expressed are displayed in a heatmap (E), with the six top canonical pathways associated with this gene-set as determined by Ingenuity Pathway Analysis (F). One-way ANOVA, $P<0.01$, Benjamini-Hochberg multiple test correction was carried out for all. *Samples were analysed under the supervision of Dr Stefania Crotta.*

The dominance of the antiviral response within the overall transcriptional response of the AECs is also clear to see when live *S. pneumoniae* was used as the secondary stimulus (Figure 17A). This gene set was further filtered for a 2-fold change of X31 + live *S. pneumoniae* co-exposure compared to X31 single exposure (Figure 17B), and X31 + live *S. pneumoniae* co-exposure compared to *S. pneumoniae* single exposure (Figure 17C). Co-exposure induced 90 genes above X31 stimulus alone, which is again far fewer than the 1007 genes induced by co-exposure in comparison to the bacterial stimulus alone. It is surprising to see that live *S. pneumoniae* exposure alone or as a co-stimulus had very little impact on epithelial responses. This could be due to the fact that D39 is a mild strain of *S. pneumoniae*. There is also the possibility that D39 may shield its PAMPs from the PRRs on the epithelia, even though bacterial TLR agonists had equally little impact on epithelia, as shown previously. A set of only 80 genes were differentially expressed by co-exposure to either virus or bacteria alone (Figure 17D, E. Appendix Table 5). Genes of note that were differentially expressed are the cytokines: CXCL2, CXCL1, and the antimicrobial peptide: Slpi (Appendix, Table 5). These 80 genes are also displayed as a heatmap (Figure 17E) and were taken for IPA (Figure 17F). The top six canonical pathways are represented. Interestingly, within these top six pathways is: ‘The role of hypercytokinemia/hypoxytokinemia in the pathogenesis of Influenza’ which further confirms the predominant role of the virus in the overall response from the AECs, and suggests that the genes induced by co-exposure may contribute to pathology.

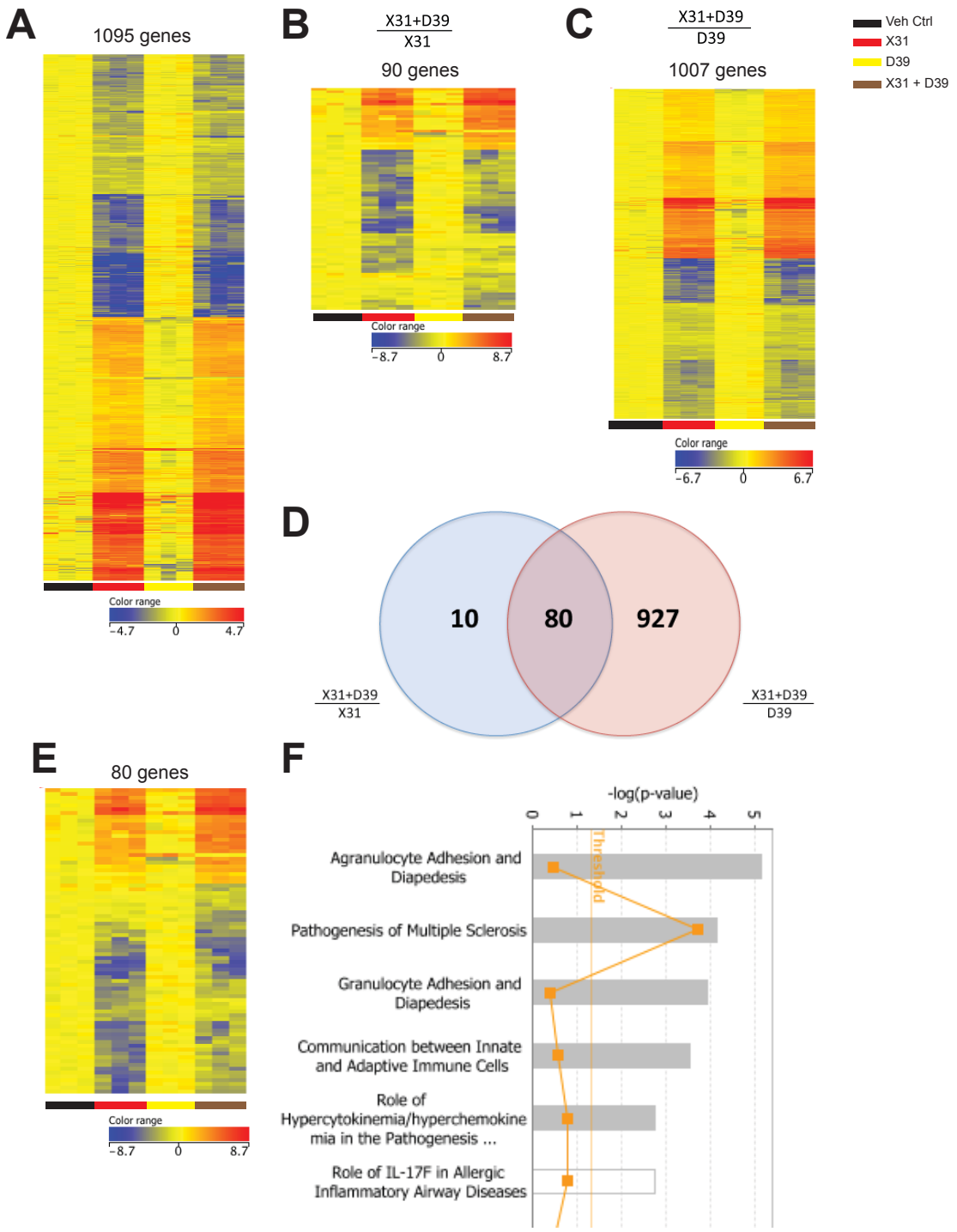


Figure 17 The antiviral response also dominates the overall transcriptional response following co-stimulation with live *S. pneumoniae*

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then with live D39 for a further 24 hours. RNA was collected for global analysis by Illumina.SingleColor.Mouse WG-6_V2_0_R0_1127 microarrays. Samples (n=3) were normalized to the median of the vehicle control group and filtered for a fold change of 2 (A). This was subsequently filtered for 2-fold change of co-stimulation versus X31 (B), or a 2-fold change of co-stimulation vs. D39 alone

(C). A Venn diagram was generated using the gene set from B and C to generate a list of genes differentially expressed in co-stimulation versus either single stimulation (D). The 80 genes most changed when X31 and D39 are used are displayed in a heatmap (E), with the 6 top canonical pathways associated with this gene set as determined by Ingenuity Pathway Analysis (L). One-way ANOVA, $P<0.01$, Benjamini-Hochberg multiple test correction was carried out for all. *Samples were analysed under the supervision of Dr Stefania Crotta.*

The mTEC system lacks any of the resident or recruited innate immune cells, which can also respond to infection by the release of cytokines, chemokines and other innate immune factors. Since, we see the virally induced genes dominating the overall AECs response to co-stimulation, whole lungs may be able to portray whether this represents the *in vivo* situation and whether the presence of the innate immune cells change the picture we find for co-exposed epithelia. Mice were either singularly infected or co-infected with X31 and D39. The primary X31 virus was given for 5 days before treatment with either PBS or D39 for 48 hours. Whole lungs were taken and processed for microarray. Samples were normalized to the average of the vehicle control group and filtered for a fold change of 4 by any of the stimuli, yielding 3173 genes differentially expressed (Figure 18A). It is evident yet again that the antiviral response dominates the whole lung transcriptional response. This gene set was further filtered for a 2-fold change in X31 + *S. pneumoniae* compared to X31 infection alone (Figure 18B), and X31 + *S. pneumoniae* challenge compared to *S. pneumoniae* infection alone (Figure 18C). Co-infection resulted in 418 genes differentially expressed compared to virus infection alone, which is once more far less than the 2721 genes differentially expressed by the co-infection over *S. pneumoniae* alone. A set of 362 genes were differentially expressed overall by both X31 and *S. pneumoniae* infection (Figure 18D, E. Appendix Table 6). Genes of note that are differentially expressed are the cytokines: CXCL2, CXCL1 and TNFα (Appendix, Table 6). All 362 genes are expressed as a heatmap (Figure 18E), and this set of genes was assessed by IPA. The top twelve canonical pathways are represented (Figure 18F). Many of these pathways are found in Figures 16 and 17. In fact ‘The role of hypercytokinemia/hypoxytokinemia in the pathogenesis of influenza’ is also found as one of the top pathways induced *in vivo* and in AECs,

further corroborating the strong cytokine induction and confirming that the overall response of the lung during co-infection is driven mainly by the antiviral response. However the differentially expressed gene list here, 362 genes are far larger than the 80 genes differentially expressed by AECs and suggest that the response from the innate immune cells contributes many gene products that the AECs do not induce.

When the three lists of genes that were differentially expressed in the three co-exposure scenarios were combined into a Venn diagram, it was surprising to find only six genes common between all three (Figure 18G, H). Interestingly, CXCL2, present twice within the gene list, and CXCL1 are both induced by all three co-exposure scenarios, which concretely confirms the findings from Figures 11-15. This indicates that the AEC response mirrors the *in vivo* response of the lung to co-infection and suggests that airway epithelia can initiate and contribute to the innate immune response during co-infection by producing proinflammatory cytokines and chemokines, essential for the recruitment of neutrophils needed to control and eliminate the bacteria but also potentially pathogenic. However, we cannot estimate how much of these cytokines and chemokines are produced *in vivo* by AECs and how important the contribution of innate immune cells are. Most likely, cross talk between the innate immune cells and the AECs and positive feed back loops through recruited immune cells producing more chemoattractants will enhance and amplify the initial epithelial response *in vivo*.

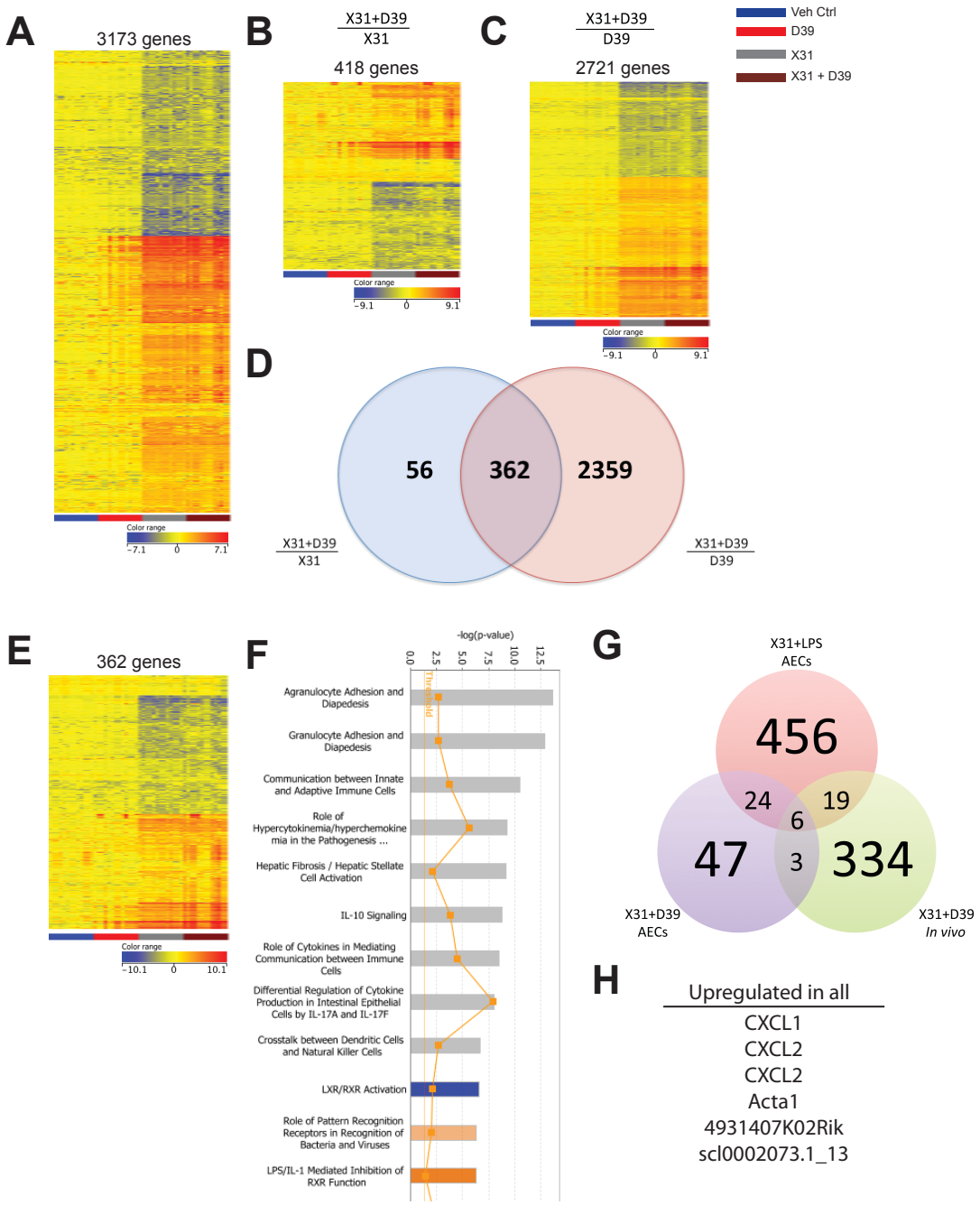


Figure 18 The antiviral response also dominates the transcriptional response by co-infected whole lungs

C57BL/6 mice were infected with $8x10^3$ $TCID_{50}$ X31 or vehicle control (PBS), then 5 days later with $2x10^7$ CFU *S. pneumoniae* (D39) or PBS. Whole lungs were collected 48 hours later. RNA was collected and prepared for global analysis by Illumina.SingleColor.Mouse WG-6_V2_0_R0_1127 microarrays. Samples (n=10) were normalized to the median of the vehicle control group and filtered for a fold change of 4 (A). This was subsequently filtered for 2-fold changes in co-stimulation versus X31 (B), or a 2-fold change in co-stimulation vs. D39 alone (C). A Venn diagram was generated using the gene set from B

and C to produce a list of genes which are commonly upregulated in co-infection compared to single infections (D). These 362 genes most changed when X31 and D39 are used *in vivo* are displayed in a heatmap (E), with the twelve top canonical pathways associated with this gene-set as assessed by Ingenuity Pathway Analysis (F). The three sets of changed genes in a co-stimulus (E, Figure 16E and Figure 17E) were generated into a Venn diagram showing only four genes differentially expressed by all three (G, H). One-way ANOVA, $P<0.01$, Benjamini-Hochberg multiple test correction was carried out for all. *Samples were collected with Dr Gregory Ellis and were analysed under the supervision of Dr Stefania Crotta.*

3.3.7 A more virulent bacterium does not induce the synergy expected

Since the transcriptional profile showed very little gene induction by the bacterium we used, a more virulent strain of *S. pneumoniae*, TIGR4, was utilised to see if that would lead to stronger antibacterial responses and induce the expected synergy. The five hypothesis driven readouts: AMPs, type I and III IFNs, ISGs, apoptosis related genes, and proinflammatory cytokines, were tested. In contrast to Figure 6, live TIGR4 induces the antimicrobial peptides: Defb3, Defb2, and S100a8, which have marginal increases following co-infection (Figure 19A).

The virus induces messenger levels of the three IFNs tested (Figure 19B). Interestingly, live TIGR4 induces IFNa4 that increases following co-stimulation, however this is not significant. Furthermore, live TIGR4 induces messenger levels of IFNb1 and IL28A. For IL28A, there is an increase following X31 with HK TIGR4 and live TIGR4, but this too is not significant.

The virus induces the three ISGs tested: Rsad2, Ifi203 and Oasl2, as expected (Figure 19C). Interestingly, the induction of IFNs by live TIGR4 alone does not correlate with an induction of the three ISGs. Similarly, the combination of X31 and live TIGR4, which resulted in induction of type I and III IFN, induced Rsad2 and ifi203, however very little induction of Oasl2 is found. Intriguingly, there appears to be suppression in the expression of Rsad2 and Oasl2 during co-stimulation in comparison to the virus alone.

There is an increase in DR5 and TRAIL induction following X31 and HK TIGR4 co-stimulation, but this is not significant (Figure 19D). TRAIL expression appears to be suppressed during X31 and live TIGR co-stimulation in comparison to X31 alone. Virus infection alone induced, although not significantly, the death receptor FAS with no further increase observed following co-stimulation,

corroborating Figure 10. It's corresponding ligand FASL however, shows an increased induction following X31 and live TIGR4 co-stimulation, but this is again not significant.

For the cytokines CXCL1 and TNF-α, there is a small induction by the bacteria alone (Figure 19E). CXCL1 is increased when X31 is combined with either HK TIGR4 or live TIGR4. An increase in TNF-α when X31 and HK TIGR4 are combined can also be observed. These increases are again not significant.

Overall, live TIGR4 stimulation of the AECs alone induces a greater response than was observed using D39. This however did not translate into stronger synergy or detectable suppressive effects when combined with X31. As these initial results did not promise to find stronger synergy, this bacterial strain was not tested further.

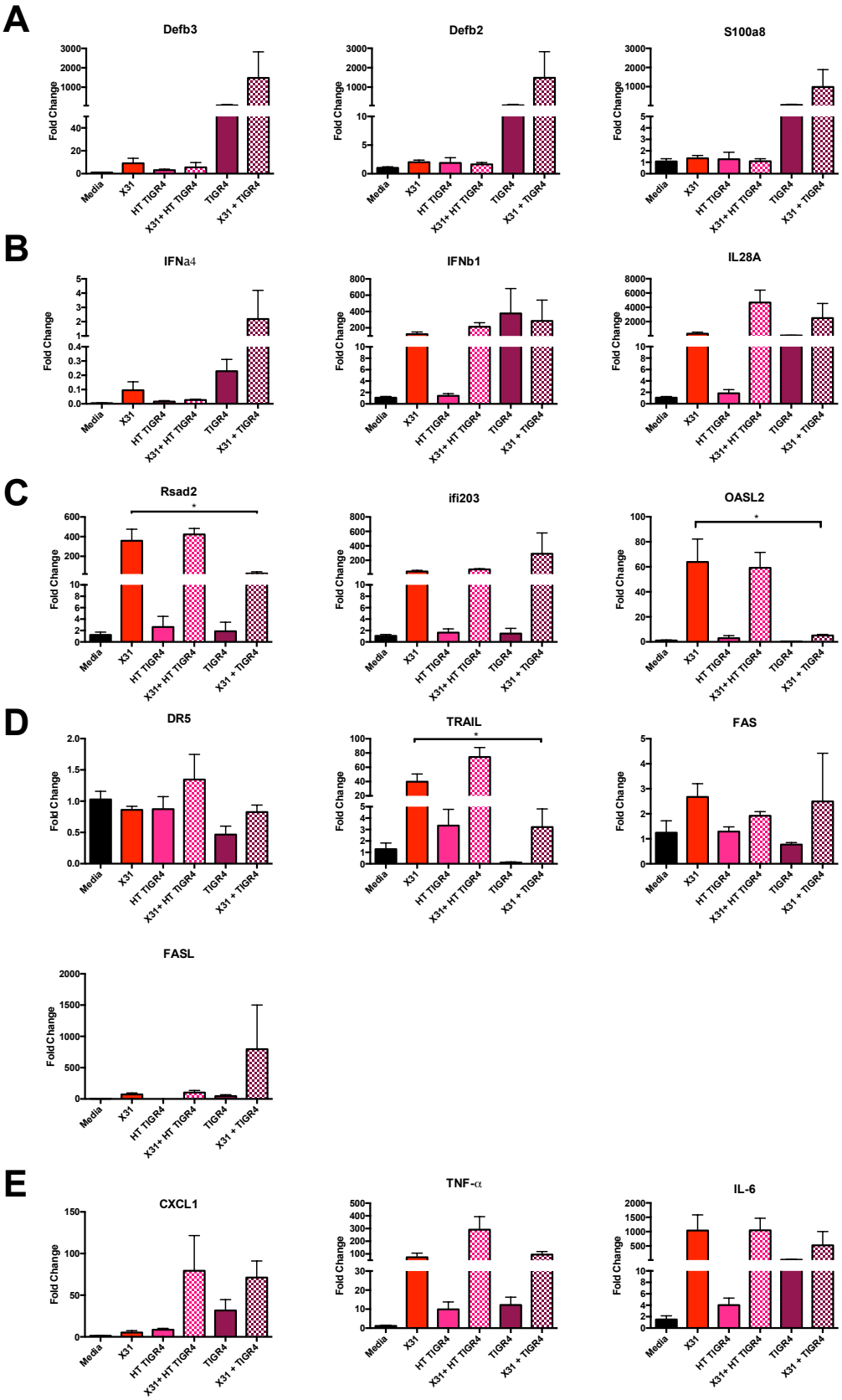

A
Defb3
Defb2
S100a8
B
IFNa4
IFNb1
IL28A
C
Rsad2
ifi203
OASL2
D
DR5
TRAIL
FAS
FASL
E
CXCL1
TNF-α
IL-6
Fold Change
Media
X31
HT TIGR4
X31+ HT TIGR4
TIGR4
X31 + TIGR4

Figure 19 Exposure to a more virulent bacterium induces more genes than D39 but does not synergise strongly with X31 exposure

C57BL/6 epithelia were infected with X31 at a MOI of 0.1 for 24 hours then with HK or live TIGR4 (10^8) for a further 24 hours. cDNA from the cultures were analysed for antimicrobial peptides (A), IFNs (B), ISGs (C), apoptosis related genes (D) and cytokines (E). Significance was assessed by unpaired t-test where * $P<0.05$. No significance for any co-stimulation:single stimulation. Graphs show mean ± SEM, n=3.

3.4 Conclusions and Discussion

It is well documented that viral and bacterial co-infection is associated with strong inflammation, lung damage, high cytokine levels and massive immune cell recruitment into the lung, leading to increased morbidity and mortality (Morens *et al.* 2008; Centers for Disease Control and Prevention (CDC) 2009; Cillóniz *et al.* 2012; Li *et al.* 2012; Niemann *et al.* 2012). Airway epithelia are one of the first and most important targets for viral and bacterial contact and invasion, and are able to produce a vast range of inflammatory cytokines and chemokines, essential for the activation and recruitment of immune cells that can help control and clear infection. We therefore tested the hypothesis of whether co-exposure of airway epithelia to the two infectious agents would lead to massive increases in the epithelial response compared to exposure to both single pathogens. This would allow us to understand better whether or not the strong immune response found in co-infection may be orchestrated by the airway epithelia. We found that the antiviral response drives the overall response of the AECs during viral and bacterial co-exposure. We did however show increased induction of proinflammatory cytokines and chemokines following co-exposure in relation to the two single stimuli. Although this airway epithelial response to co-exposure may contribute to the overall innate immune response, it is unlikely that it is the epithelium alone that drives the massive proinflammatory cytokine signature observed *in vivo* during co-infection.

The exposure timings employed in our *in vitro* model greatly differ from those used *in vivo*. We infect the mTECs with X31 for 24 hours, and then they are co-exposed with TLR agonists or bacteria for a further 24 hours before samples were taken. *In vivo,* bacteria are given 5 days after the primary IAV infection, with samples taken 24 hours later and onwards. A huge antiviral response occurs within

these first 5 days, which allows the lung to potentially clear the first viral infection before the second bacterial one is given. The bacteria therefore enter into an already inflamed lung. We could not do this *in vitro*, as the virus would kill most, if not all of the AECs within 5 days. Our system is therefore rather a co-exposure as the virus and bacterial stimuli are given concurrently, which may or may not occur *in vivo*. This may be one reason for why we did not confirm many of our hypotheses.

We first hypothesized that the primary viral infection would suppress the production of AMPs, which could contribute to the bacterial outgrowth observed in co-infected mouse lungs (Ellis *et al.* 2015). We found that surprisingly the bacterial stimulation alone rarely induced any of the antimicrobial peptides, which made subsequent inductions or suppression difficult to determine. This may be due to the bacteria shielding their PAMPs, which may only become visible once phagocytosed and digested. AECs do not phagocytose bacteria, nor do the bacteria infect the AECS. Therefore, presentation of PAMPS to the AECs may be more limited to innate immune cells such as macrophages. However, if insufficient TLR activation by bacteria were the case then both TLR agonists should be inducing the expression of these AMPs, but generally they do not. We only found AMP induction using a more virulent *S. pneumoniae* strain, but AMP induction was neither enhanced nor suppressed by viral co-stimulus.

AECs upregulated secretory leukocyte protease inhibitor (Slpi) in response to co-stimulation (Figures 16, 17. Appendix Tables 4, 5). It has been documented to be produced by the epithelia of the lung (Abe *et al.* 1991), and its role as an antimicrobial peptide has been described (Fahey and Wira 2002). Interestingly, calgranulin A (S100a8) and calgranulin B (S100a9) are differentially expressed

during X31 and TLR4 co-stimulation (Figure 16D and E, Appendix Table 4). These AMPs form a heterodimer to form calprotectin which has been shown to have microbicidal activity against many bacteria (Abtin *et al.* 2010), by inhibiting bacterial adhesion to mucosal epithelium and bacterial growth through zinc chelation. S100a8 and S100a9 are induced at high levels within the first 24 hours after bacterial infection *in vivo* (Raquil *et al.* 2008). They are also known to be upregulated by the cytokines IL-1α, IL-6, TNF-α, some of which have been found to be significantly increased by viral and bacterial co-exposure of the mTECs. S100a8 and S100a9 not only show bactericidal properties but have also been described to have chemotactic and proinflammatory properties. Treatment of mice with anti-S100a8 and anti-S100a9 antibodies had no effect on bacterial load or survival, but did however cause a reduction in the recruitment of neutrophils and macrophages (Raquil *et al.* 2008). The three defensins Defb1, and Defb3 show an increase in messenger levels following certain co-stimulations. Defensins have also been characterised to be chemotactic for monocytes and neutrophils (Raquil *et al.* 2008).

The increase in expression of these AMPs contradicts our hypothesis of suppression during co-exposure. This may suggest that during co-stimulation, the virus does not hinder their expression. It may also be possible that their increases are contributing to the proinflammatory and immune cell recruitment responses of the AECs rather than acting as conventional AMPs. However, we have assessed only expression levels of these few AMPs, and therefore cannot conclude whether the same is true for all AMPs.

As we saw no great indication of AMP production from the AECs following any sequence of stimulation we decided not to pursue this any further. As we only measured transcriptional induction of these AMPs it may be useful to measure

protein levels before drawing concrete conclusions, and direct quantification of bactericidal properties of AEC supernatants might allow for a functional assessment of antibacterial activity without prior knowledge of the mechanisms involved. Innate immune cells also have the ability to express antimicrobial peptides, with neutrophils known to express S100a8 and S100a9 (Raquil *et al.* 2008), and there is the possibility that immune cells are the major contributor of these molecules in the lung. This would be in contrast to reports in the gut of strong AMP production by epithelial cells. Alternatively, immune-cell derived cytokines such as IL-22 or other, maybe cell contact-dependent signals, may be required on top of bacterial stimuli to induce AMP induction in lung epithelia. Therefore, a co-culture system may be useful to determine the effect of the innate immune cells on the airway epithelial production of antimicrobial peptides. As greater effects are seen *in vivo* compared to *in vitro*, immunofluorescence detection of antimicrobial peptides on lung sections may show directly whether airway epithelia produce these peptides.

We next hypothesized that co-stimulation of the AECs would result in increased production of type I and III IFNs and subsequently ISGs. We found that the induction of IFNs and ISGs were mainly driven by the antiviral response and remained unchanged following co-stimulation. IFN production is traditionally associated with antiviral immunity, however the role of type I IFNs in *S. pneumoniae* infections have also been studied (Parker *et al.* 2011; Koppe *et al.* 2012). IFNβ was been shown to be produced following *S. pneumoniae* infection (Koppe *et al.* 2012). The role of type I IFN during bacterial infections is controversial. One study which utilised mice lacking the *Ifnar* gene showed increased susceptibility to disease, where the mice succumb to *S. pneumoniae*

bacteraemia (LeMessurier *et al.* 2013). In contrast, other studies showed that *Ifnar-/-* mice were more resistant to Listeria infection (Auerbuch *et al.* 2004), and type I IFN promoted bacterial infection and pathology (Carrero *et al.* 2004; O'Connell *et al.* 2004; Osborne *et al.* 2016). Listeria is an intracellular pathogen whereas *S. pneumoniae* is extracellular and so cannot be directly compared. Therefore, it is not yet known whether type I IFNs are protective or pathogenic during IAV-*S. pneumoniae* co-infections but Shahangian *et al.* propose that type I IFNs contribute to lack of bacterial control (Shahangian *et al.* 2009).

IFNa4 messenger levels were not induced by either the virus or bacterial stimulus, but this is just one of 11 subsets of IFNα within a mouse and hence the right IFNα may not have been tested. Nevertheless, an induction in IFNα proteins as tested by ELISA was found for the virus and for the HK and live *S. pneumoniae* (D39) stimuli. This discrepancy may be due to early induction of IFNa4 which is not detectable anymore by mRNA levels at the time of testing. There is rarely any significant increase in type I or III IFNs during co-stimulation in comparison to the two single stimuli; their induction is rather driven by the virus alone. Given the lack of an increase in production of type I and III IFNs following co-stimulation, it is not surprising that there is also no significant increase in ISG production. ISGs are driven also by the antiviral response, which is further confirmed in the AEC transcriptional profiles.

This dominance of the virus stimulus for IFN and ISG induction is hardly surprising since IFNs and ISGs are predominately an antiviral response potently induced by the RIG-I signalling, and less so by bacterial PRRs. It has been reported that the pneumolysin of *S. pneumoniae* interacts with TLR4, which activates TRIF to induce type I IFN (Malley *et al.* 2003; Srivastava *et al.* 2005;

Parker *et al.* 2011). However, one study suggests it is not the TLR4 pathway that is required to initiate an immune response to *S. pneumoniae*, but rather the NLRP3 pathway (McNeela *et al.* 2010).

Our results of no significant induction of type I IFNs following co-stimulation disagrees with our hypothesis and the findings from Nakamara and associates, who showed a synergistic increase in type I IFNs following co-infection (Nakamura *et al.* 2011). The Nakamara *et al.* study however reverses the order of infection by giving *S. pneumoniae* before the virus, PR8 - the more virulent strain of influenza. This completely changes the response to the two infections and as such cannot be directly compared to our study. Nonetheless, the lack of significant amplification of IFNs and ISGs may be due to the different timings discussed earlier. Peak IFN levels are usually seen 4 hours following infection whereas we measured the IFNs and ISGs 48 hours after the initial viral infection and 24 hours after the secondary stimulus. The levels of IFN may therefore be decreased and the ISGs may have already reached plateau. Furthermore, the lack of innate immune cells such as pDCs, which are potent IFN producers, may also explain the lack of increased production during co-stimulation. Koppe *et al.* suggested that it is not the lung epithelial cells themselves which produce type I IFNs after S. pneumoniae infection but are activated to induce ISGs by type I IFN produced by macrophages in a paracrine manner (Koppe *et al.* 2012). The contribution of innate immune cells to the production of type I IFN may also explain the synergistic increases observed in the Nakamara *et al.* study. Future studies could employ a co-culture system and test earlier for IFN and ISG production. Overall, these results suggest that it is not the AEC response alone that is driving increased IFN production within co-infection.

Primary viral infection causes damage within the lung, which can be mediated by the cytotoxic effects of the virus or by the antiviral response. We therefore hypothesised that the induction of apoptosis related genes would be increased following co-stimulation in relation to the two single stimuli. Type I IFN has been described to cause damage to the lung via DR5-TRAIL interaction following IAV infection (Herold *et al.* 2008; Davidson *et al.* 2014) and it is via this signalling pathway that the outgrowth of the secondary bacteria is facilitated (Ellis *et al.* 2015). The airway epithelia do upregulate both type I and type III IFNs following IAV, however there was no corresponding induction of DR5 on the epithelia. Neither bacterial stimulation nor co-stimulation induced DR5. FAS, another apoptotic receptor on the epithelia was also not induced by any stimulation (Waring and Müllbacher 1999). The lack of DR5 or FAS upregulation may be due to type I IFN indirectly causing DR5 expression through the induction of cytokines that are not present within mTEC cultures. Again this could be explained by a contributing factor missing from the cultures. Innate immune cells may be providing this contributing factor, which may be soluble or provided via cell-cell contact, and a co-culture system may be able to address this question.

On the other hand there is a significant increase in their corresponding ligands: TRAIL and FASL. TRAIL can be expressed by a variety of cell types such as B cells, DCs, gut epithelia, and macrophages (Wiley *et al.* 1995). FASL is a transmembrane protein which can be cleaved to be a soluble ligand (Hohlbaum *et al.* 2000). This suggests that when death receptors are induced, AECs may contribute to the increased induction of apoptosis and lung damage. However the increased epithelial cell death is not orchestrated by the AECs alone. Since we saw no induction of death receptors we did not test further for the induction of apoptosis.

Future experiments could however test for apoptosis induction and integrity of the epithelium by quantifying bacterial migration through the epithelium, or tracking the transepithelial electric resistance (TEER) levels throughout the experiment. Another way to quantify damage of the epithelia is by measuring the levels of caspase since this an important mechanism of cell death.

The massive increase in innate immune cell recruitment along with proinflammatory cytokine production within co-infected mouse lungs lead us to next hypothesise that this is orchestrated by the AECs. Neutrophils and other phagocytic cells in co-infection are essential for the control and elimination of bacteria. The airway epithelia do very clearly induce significantly increased amounts of CXCL1 and CXCL2 in response to a co-exposure versus during single exposure, which could correlate with the increased neutrophil numbers found *in vivo* (Ellis *et al.* 2015). These findings also disagree with studies that found the CXCL1 and CXCL2 were blocked in co-infection by type I IFNs (Shahangian *et al.* 2009; Schliehe *et al.* 2014), as IFN is clearly present in these cultures. It could be that IFN-mediated CXCL1 and CXCL2 suppression is more prevalent in immune cells whose chemokines may contribute strongly to the overall neutrophil recruitment into the lung. Schliehe *et al.* showed that Setdb2, a type I interferon-stimulated protein, repressed CXCL1 expression which in turn had a negative effect on early neutrophil recruitment, tissue damage and bacterial burden during IAV-*S. pneumoniae* co-infection (Schliehe *et al.* 2014). This study therefore highlights the protective role of early CXCL1 signalling and neutrophil recruitment. TNF-α which is essential in during IAV - *S. pneumonia* co-infection for bacterial control and clearance was also found to be significantly induced by the AECs thus contributing to the beneficial innate immune response (Ellis *et al.* 2015). However,

high concentrations for prolonged periods of these cytokines and chemokines may have the potential to be pathogenic. TNF-α and activated neutrophils can induce apoptosis of epithelial cells, which may contribute to lung injury and disease outcome (Abraham 2003; Chau *et al.* 2004; Moraes *et al.* 2006).

Many other cytokines and chemokines do appear to be more highly expressed in co-exposure such as CCL5, GMCSF, GCSF, IL-1β and IL-1α that function to recruit and activate phagocytic cells to eradicate the pathogens and potentially contribute to pathogenesis. IL-6 was not significantly induced when HK *S. pneumoniae* was used as the secondary stimulus, and interestingly there appears to be a suppression of this cytokine when the live bacteria is used during co-stimulation. This could be due to a late peak in mRNA that has not been translated at the time of measuring. On the other hand, a protease, SpyCEP, released by *Streptococcus pyogenes* has been described to inhibit chemokines (Zingaretti *et al.* 2010). *S. pneumoniae* could therefore potentially be releasing similar proteases which are cleaving cytokines and chemokines, although this remains to be proven. Nonetheless, IL-6 was significantly increased in co-exposure with TLR agonists compared to the two single exposures. IL-6 has both pro- and anti-inflammatory roles, with one study reporting its role in neutrophil survival during influenza infection (Dienz *et al.* 2012). Therefore, this increased response to co-stimulation could be acting in tandem with CXCL1 and CXCL2 to recruit and promote the survival of neutrophils, which, as discussed, may or may not be protective. One study demonstrated that the control of inflammation was essential for the protection of mice during co-infection (Damjanovic *et al.* 2013). This however was only effective if the bacterial infection was controlled by antibiotic treatment. This highlights the requirement of the induction of these proinflammatory

cytokines and chemokines for the control of the bacteria, however an unchecked inflammatory response along with an outgrowth of bacteria can severely damage the lung.

Overall, the AECs can contribute to the induction of proinflammatory cytokines and chemokines which induce the recruitment of the innate immune cells. It is unlikely that it is the AEC response alone that is responsible for the documented massive increase of immune cells and proinflammatory cytokines in co-infection. We find however that the increased epithelial response *in vitro* to co-stimulation closely reflects the stronger *in vivo* response to co-infection. In particular, data within the lab found that after IAV-*S. pneumoniae* co-infection *in vivo*, showed similar increases in these proinflammatory cytokines and chemokines. The different timings of co-exposure *in vivo* and *in vitro*, as discussed earlier, however render direct comparison between *in vivo* and *in vitro* results difficult.

Since it was thought that D39 might be a poor stimulator of the epithelium, the more virulent strain TIGR4 was also used. TIGR4 stimulation induced AMPs far greater than TLR agonists or D39. However, comparisons between single stimuli and co-exposure did not show the same significant synergies as when D39 was used. TIGR4 is a capsulated virulent serotype 4 strain, whereas D39 is a capsulated clinical serotype 2 strain of *S. pneumoniae* (Lanie *et al.* 2006). Whether these strains keep or lose their capsule during growth and within the mTEC culture is not known. Variability in the expression and accessibility of surface proteins resulting from either keeping or losing their capsule may explain the differences in the AEC responses (Bogaert *et al.* 2005). Major genetic differences have been found between the two strains such as a single sortase gene in D39 compared to four in TIGR4 (Tettelin *et al.* 2001; Lanie *et al.* 2006). Such genetic differences may

also explain the divergence in disease outcomes *in vivo* and why the same synergistic effects are not observed when the same genes were analysed.

The antiviral response dominates the overall innate immune response of the airway epithelia to infection. However, it has been possible to identify significant synergies in the AEC response to co-stimulation. AECs do appear to contribute to the proinflammatory cytokine and chemokine responses seen *in vivo*. These chemokines are involved in recruiting neutrophils whose antibacterial function is crucial in co-infection (Ellis *et al.* 2015). Co-exposure of co-cultures may however offer a more realistic scenario, or migration assays could be set up to test if increased chemoattractant production by co-exposed epithelia translates into stronger immune cell recruitment.

In conclusion, the epithelial barrier is crucial to protect the host from the outside environment and many different invading pathogens. The airway epithelia do respond to both viral and bacterial stimulation, and the increased levels of some apoptosis related peptides, cytokines and chemokines observed following co-stimulation agrees with previous findings. We find evidence for increased proinflammatory activity of co-exposed epithelia, and while these effects are less strong than those observed *in vivo*, epithelia might be important to initiate a stronger inflammatory response in co-infection which is subsequently amplified by positive feed back loops such as more chemokine production by the recruited innate immune cells. Therefore, airway epithelia may not be the main driving force but the initiator of the documented massive increase in inflammation, lung damage and immune cell recruitment during co-infection that is associated with morbidly and mortality.

Chapter 4. Airway epithelia from IAV susceptible 129S8 mice differ from C57BL/6 epithelia in responses, cell composition and regeneration

4.1 Background

Differences between hosts in influenza severity or protection can be attributed to varying adaptive immune response depending on the history of previous exposures, to the virus strain's virulence, or to defined medical preconditions that represent risk factors for severe influenza. However, apart from these differences, there may also be a role in the host-dependent variations in anti-influenza innate immune responses. Growing evidence indicates that lung damage and epithelial cell death in influenza depends on immune-mediated effects. Fatal disease may therefore be a result of pathology associated with an excessive immune response (Tisoncik *et al.* 2012), and the degree of the immune response may be at least partly determined by host genetics.

Recently, host genetics have gained momentum as playing a major role in the outcome of disease (Horby *et al.* 2012). In humans, a correlation has been made between MHC haplotypes and the outcome of infectious disease (Blackwell *et al.* 2009; Ruiz-Hernandez *et al.* 2016). Specifically in influenza, a study by Albright and associates found that in a specific population from Utah, USA, there was an inheritable predisposition to influenza mortality. Examining the genealogy of this population indicated that a close or distant relation of an individual who had succumbed to influenza infection in the past, was more likely to succumb themselves (Albright *et al.* 2008). The familial clustering of the HPAI H5N1 and H7 N9 has furthermore increased the awareness of genetic susceptibility to infection

(Ungchusak *et al.* 2005; Kandun *et al.* 2006; Sedyaningsih *et al.* 2007). Fortunately, the virus as of yet has not displayed efficient human-to-human transmission (Xiao *et al.* 2014). 90% of the reported human-to-human transmission cases that have occurred are between genetically related family groups (Hu *et al.* 2014).

Genetic predisposition to disease has also been confirmed in animal studies. Similarly to humans, chickens displayed a strong association with the MHC haplotype and resistance or susceptibility to pathogens (Banat *et al.* 2013). Hernandez and associates showed two genetically different in-bred lines of chickens, which diverged in the outcome of infection, with one resistant and the other susceptible. Their resistance to infection was independent of the adaptive immune response, however the innate immune response was not studied (Ruiz-Hernandez *et al.* 2016).

Susceptibility to IAV also varies between inbred mouse strains. An important influenza restriction factor that varies between mouse strains is Mx1. Susceptible mice have a nonsense point mutation or deletions in the *Mx1* genes resulting in non-functional Mx1 protein (Staeheli *et al.* 1988). Mx1 is a potent ISG capable of inhibiting IAV replication (Lindenmann 1962; Haller *et al.* 1979; Staeheli *et al.* 1988). Further mouse studies have confirmed that host genetic background determines susceptibility to IAV-induced disease with susceptibility correlating with high viral loads, excessive inflammation and severe lung damage (Boon *et al.* 2009; Srivastava *et al.* 2009; Blazejewska *et al.* 2011; Trammell *et al.* 2012). Our lab utilized a resistant mouse strain – C57BL/6, and a susceptible mouse strain - 129SvEv, and showed that differences in the innate immune response to IAV played a direct role in the outcome of disease. High type I IFN levels correlated with disease severity where the viral loads remained unchanged. Susceptible

129SvEV mice that lacked the IFNαβ receptor displayed reduced morbidity and mortality, confirming the contribution of excess IFNαβ to disease. High levels of IFNαβ also correlated with high levels of DR5 on epithelia and TRAIL on inflammatory monocytes, whose interaction resulted in lung epithelial damage (Herold *et al.* 2008; Davidson *et al.* 2014). This pathogenic potential of type I IFNs is in stark contrast to the potent antiviral abilities associated with this family of cytokines (Isaacs and Lindenmann 1957; Garcia-Sastre *et al.* 1998; Koerner *et al.* 2007). Type I IFNs however are known to induce many other cytokines, which contribute to the hypercytokinemia or 'cytokine storm' that has been well documented from past pandemic IAV strains (de Jong *et al.* 2006; Peiris *et al.* 2009; Mauad *et al.* 2010).

Since IAV preferentially replicates within the airway epithelium of the lung, and some of the first cytokines to be released by the epithelia post IAV infection are the type I IFNs (Crotta *et al.* 2013), the host genetic background could determine at the level of the epithelia how the host response initiates and thus the outcome of disease.

The lung is a complex organ consisting of an upper respiratory tract – nasal cavity and trachea, and a lower respiratory tract – bronchioles and lungs. Since we breathe in a plethora of microbes every day, the immune system of the lung is vital in protecting the host from disease. The airway epithelium plays an essential role in this defence. Not only must the airway epithelium respond to an infection by recruiting and activating the innate and adaptive immunity, it must also repair itself from such exogenous and endogenous insults and injury.

The lung is a highly quiescent organ which was traditionally associated with a low cell turnover. However, upon injury, lung epithelia can proliferate rapidly to

repair damage and return to homeostasis (Blenkinsopp 1967; Rawlins and Hogan 2006). The tracheal epithelium consists of three major cell types: basal, secretory, and ciliated cells. The basal cells are progenitor cells for the airway epithelium, capable of self renewing and differentiating into both secretory and ciliated cells (Rock *et al.* 2009; Whitsett and Alenghat 2015). Basal cell proliferation is important following damage to the lung, such as that initiated by IAV infection, to repair the lung and return back to its pre-infection state, allowing the host to recover. Secretory cells such as club cells produce mucus and other proteins able to trap and potentially kill pathogens. Ciliated cells constantly move in a co-ordinated fashion to expel any invading pathogens from the lung. Studies have also shown the potential of the secretory club cells to self renew, differentiate into ciliated cells and dedifferentiate back to basal cells (Donnelly *et al.* 1982; Breuer *et al.* 1990; Watson *et al.* 2015).

In some cases, proper repair and regeneration following damage does not occur but rather a remodelling of the lung takes place, resulting in in fibrosis that is associated with a spectrum of chronic lung diseases such as: Interstitial pneumonitis (IPF), ciliopathies, cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD) (Chilosi *et al.* 2002; Randell 2006). In ciliopathies, a defect in the ability of the cilia to move or even a lack of cilia can occur (Waters and Beales 2011). COPD and cystic fibrosis often associate with increased mucus production due to goblet cell metaplasia (Rock *et al.* 2010). Such chronic lung diseases have also been associated with a hereditary component (Marciniak and Lomas 2010) pointing to a possible genetic link to remodelled airway epithelia. Any deviation of the repair process from rapidly reaching the pre-injury homeostasis of the lung will

greatly impact the host's ability to recover fully from an infection, resulting in increased morbidity and mortality or in long-term alterations of the lung.

Given that genetic differences determine the host's ability to detect, respond to, and eliminate infections, all of which correlates with the outcome of disease, it remains to be elucidated whether such genetic differences determine responses to infection at the level of the airway epithelium.

4.2 Hypothesis and Aims

The airway epithelium is the first host target of IAV infection. It can respond to this infection by orchestrating the innate and adaptive immune response to clear the virus, and it must repair any infection-related damage. Differences in the host genetic background have been associated with disease outcome and could be playing a role in epithelial responses and repair. To test the contribution of genetic differences in airway epithelia to the outcome of disease caused by IAV, epithelia from different in-bred mouse strains were utilised to quantify the response to infection and the regenerative capabilities. Primary mouse tracheal epithelia from resistant low IFN and susceptible high IFN responders were used for this.

I hypothesize that:

- The high and low IFN response found *in vivo* will be recapitulated by the corresponding mouse airway epithelia, resulting in higher IFN production from the 129S8 AECs compared to C57BL/6 AECs
- The 129S8 epithelia, due to inducing more IFNs, will consequently express higher levels of ISGs
- 129S8 epithelia may have increased cytokine and chemokine production contributing to the cytokine storm found *in vivo* in 129 mice
- Alternatively or additionally, the epithelium of the susceptible 129S8 mouse strain may be less capable of repairing

4.3 Results

4.3.1 129S8 mice are not high IFN responders *in vitro*

129SvEv and other sub strains of 129 mice, such as 129S8, were found to be more susceptible to influenza disease, which correlated with high type I IFN levels in the lung. Upon infection, airway epithelia release type I IFNs, thus 129S8 epithelia could be producing more IFN than their resistant counterpart epithelia from C57BL/6. However, following infection of the two genotypes of epithelia this phenomenon was not discovered, but rather the opposite (Figure 20). IFNa4 messenger levels were not induced 6, 24 or 48 hours post infection by epithelia of either genotype (Figure 20A). This is only one of 11 subsets of IFNα in mice and thus the right IFNα may not be tested here. There is an induction in protein levels however by C57BL/6 epithelia at both 24 and 48 h.p.i. This is not recapitulated in the 129S8 epithelia which do not appear to be producing IFNα in response to infection, resulting in a significant difference between the two genotypes at both time points (Figure 20B). IFNb1, another type I IFN, was also quantified. For this IFN, a significant induction by C57BL/6 epithelia at 24 h.p.i can be observed, but at no other time-points. 129S8 epithelia again display no mRNA induction of type I IFNs (Figure 20A). IFNβ protein production by C57BL/6 epithelia is only detectable at 48 h.p.i, whereas again it is not produced by the 129 epithelium (Figure 20B). As the epithelium of the 129S8 mice do not seem to be producing any type I IFNs, the type III IFNs were tested, which are known to be expressed by the epithelia and play a redundant role with type I IFNs in epithelial cells (Crotta *et al.* 2013). Both C57BL/6 and 129S8 epithelia do induce messenger of IL-28A, albeit a significantly more robust induction by C57BL/6 than by 129S8 epithelia (Figure 20A). IFNλ

protein levels are more strongly induced by C57BL/6 epithelia at both 24 and 48 h.p.i, however only minimal amounts are detectable by 129S8 at 48 h.p.i.

Overall, this indicates that the *in vivo* phenotype is not recapitulated in airway epithelia *in vitro.* C57BL/6 generally induce significantly more IFNα, IFNβ and IFNλ than the 129S8 epithelium. The low production of IFNs from the 129S8 AECs could be due to the lack of innate immune cells within the culture, such as pDCs which are known to be potent type I IFN producers and show differential IFN production between the mouse strains in question (Asselin-Paturel *et al.* 2003; Davidson *et al.* 2014). IFNλ induction is far greater than any type I IFN induction, which could indicate a greater role for IFNλ within the epithelium against influenza infection.

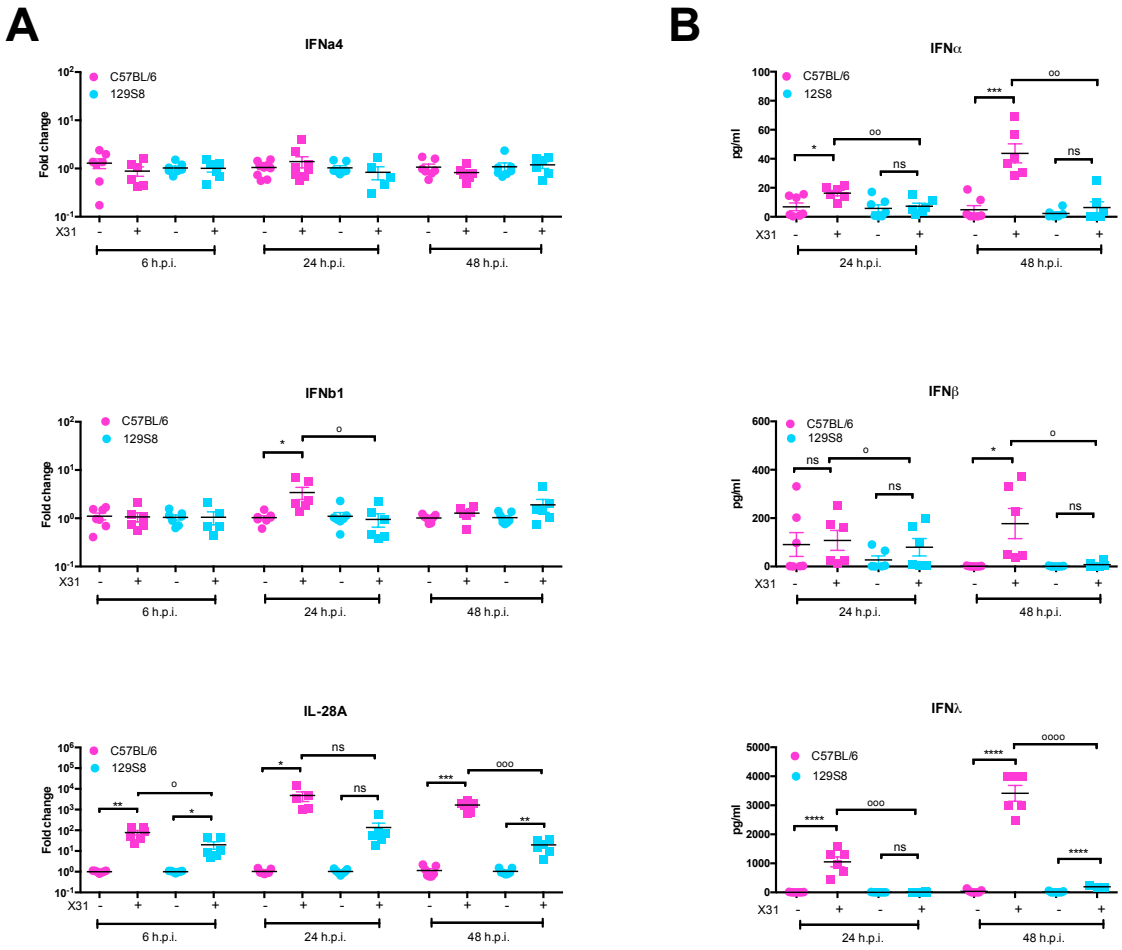


Figure 20 C57BL/6 epithelia are greater type I and III IFN producers compared to 129S8 epithelia

C57BL/6 and 129S8 epithelia were infected with X31 at a MOI of 0.1 for 6, 24 and 48 hours. cDNA from the cultures were analysed for IFNa4, IFNb1 and IL-28A (A). Supernatants were collected after 24 and 48 hours and quantified for IFNα, IFNβ and IFNλ (B). Significance was assessed by unpaired t-test where * represents X31 infected:corresponding media ctrl, and ^{o} represents C57BL/6:129S8. * P<0.05, ** P <0.01, *** P< 0.001 and **** P< 0.0001 Graphs show mean ± SEM. Data pooled from two independent experiments, n=6-11.

4.3.2 129S8 epithelium are more responsive to infection than C57BL/6 epithelium

Although the 129S8 epithelium only induced small amounts of IFNs, the induction of ISGs was tested nonetheless. Davidson and colleagues found that susceptibility follows the stromal IFNAR1 genotype, with chimeric mice more susceptible to influenza virus only if the stromal cells express the IFNαβ receptor (Davidson *et al.* 2014). Therefore, it may not be the amount of IFNs released but rather the responsiveness of the epithelia to immune-cell produced IFNs, which may be different between 129 and B6 epithelia and could determine susceptibility. Upon stimulating both C57BL/6 and 129S8 epithelia with equivalent doses of type I (IFNβ) and type III (IFNλ), as determined by titration (shown in Chapter 6, Figure 38), there is a significant induction in the epithelia of both genotypes in messenger levels of Rsad2, Oasl2 and Ifi203 (Figure 21A). Intriguingly, for Rsad2 and Oasl2, 129S8 epithelia have a significantly higher expression than C57BL/6. Although there is a higher expression of Ifi203 by 129S8 epithelia upon stimulation with IFNβ, this is not significant.

To verify if this occurs following a proper viral infection, C57BL/6 and 129S8 epithelia were infected with X31 and levels of matrix, a viral protein used to measure viral replication, and ISG levels were analysed after 6, 24 and 48 hours (Figure 21B). For C57BL/6 epithelia, there appears to be a proliferation of the virus between 6 and 24 hours, which declines at 48 hours, whereas the viral matrix expression slowly declines from 6 hours onwards within the 129S8 epithelia. An induction of all three ISGs in both genotypes can be observed following IAV infection. Furthermore, 129S8 epithelia are generally expressing significantly more of the three ISGs, specifically at 48 h.p.i. This higher expression in ISGs by 129S8

epithelia could explain the discrepancy in matrix levels, since higher ISG levels induced early, as seen with Rsad2 at 6h.p.i, could be indicative of a stronger or more rapid antiviral response that reduces virus and therefore the matrix levels. However, this is just a small subset of the hundreds of ISGs induced by IAV infection and thus these along with viral titres would need to be quantified before concrete conclusions can be drawn. These results nonetheless indicate that 129S8 epithelia, although not higher IFN producers, are in fact higher IFN responders in the production of ISGs.

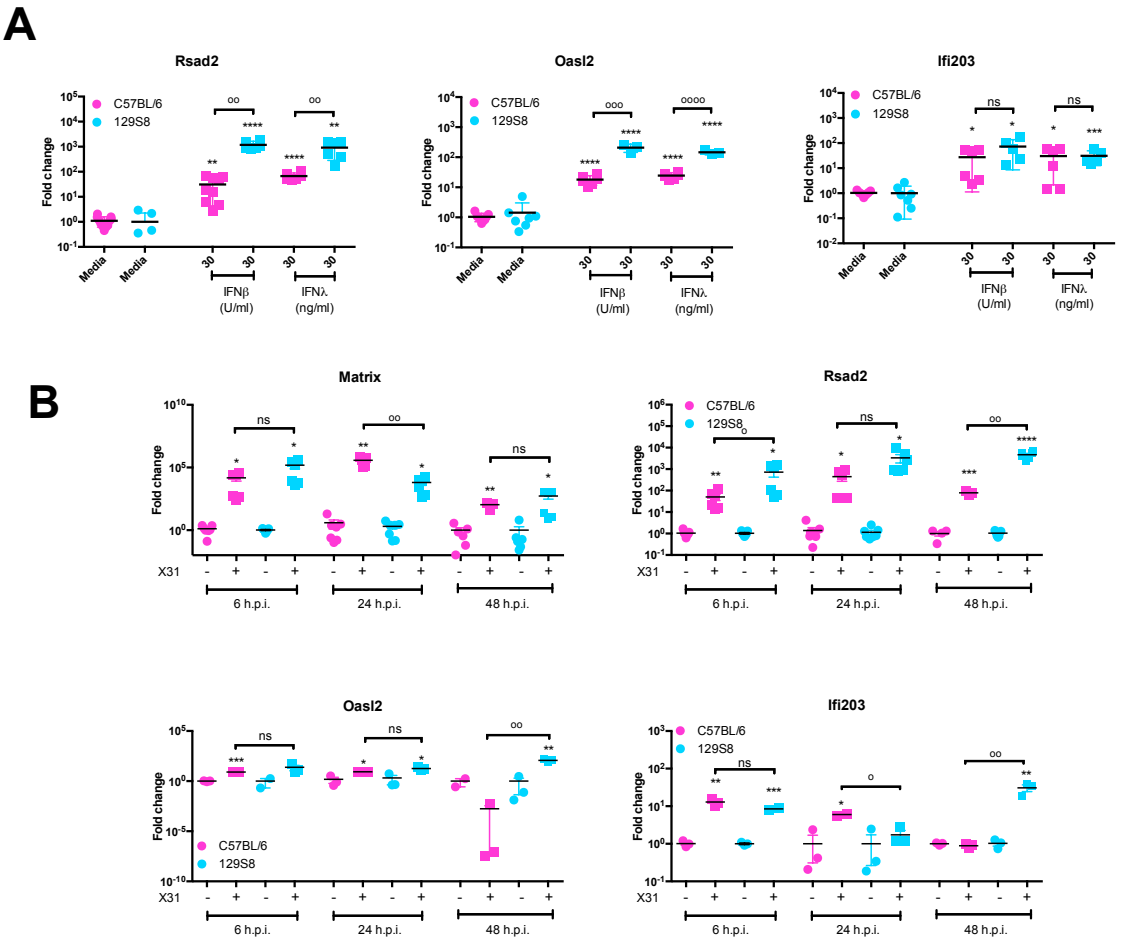


Figure 21 129S8 AECs respond more strongly to stimuli by expression of ISGs than C57BL/6 AECs

C57BL/6 and 129S8 epithelia were treated with media control or IFNβ (30 U/ml), or with IFNλ (30 ng/ml) for 4 hours. cDNA from the cultures were analysed for Rsad2, Oasl2 and ifi203 (A). C57BL/6 and 129S8 epithelia were infected with X31 at a MOI of 0.1, or media control for 6, 24 and 48 hours. cDNA from the cultures were analysed for Matrix, Rsad2, Oasl2 and ifi203 (B). Significance was assessed by unpaired t-test where * represents induction:corresponding media ctrl, and ^{o} represents C57BL/6:129S8. * P<0.05, ** P <0.01, *** P< 0.001 and **** P< 0.0001 Graphs show mean ± SEM. Data pooled from three independent experiments, n=3-11.

Hypercytokinemia and hyperchemokinemia have been well established in severe influenza infections and are associated with increased mortality (de Jong *et al.* 2006). Therefore, 129S8 epithelia were tested for increased responsiveness to IAV infection by expression of cytokines and chemokines, compared to C57BL/6 epithelia (Figure 22). 129S8 epithelia showed increased levels of CXCL1 expression throughout infection, whereas C57BL/6 epithelia appear to induce CXCL1 only at 24 h.p.i. The higher expression of CXCL1 by 129S8 at 48 h.p.i. is statistically significant. TNF-α seems to be induced at similar levels by both genotypes at 24 h.p.i, however at 48 h.p.i. the 129S8 epithelia are inducing significantly more than C57BL/6 epithelia. For IL-6, 129S8 epithelia have a statistically significant increased expression at 6h.p.i compared to C57BL/6 epithelia, however the levels are similar at 24 and 48 h.p.i. The expression of CXCL2 remains similar between the genotypes at 6 and 24 h.p.i, whereas at 48 h.p.i. there is a greater expression in 129S8 epithelium. This indicates that 129S8 have a higher expression of chemokines and cytokines following IAV infection, compared to C57BL/6 mice. However, these are a small set of cytokines and chemokines. Furthermore, protein levels may give a better indication of whether the cells are actually producing these cytokines, hence a cytokine multiplex would give a better overview of whether 129S8 epithelia are more responsive to infection in terms of cytokines and chemokines.

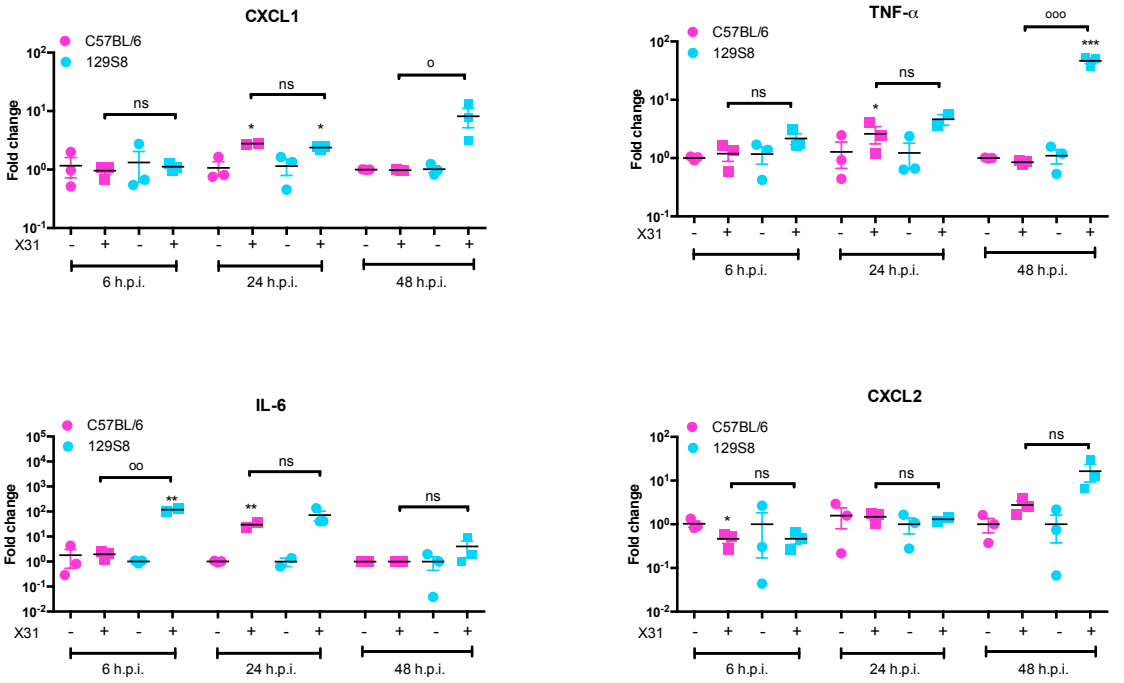


Figure 22 129S8 AECs have a higher expression of some cytokines after IAV infection, compared to C57BL/6 AECs

C57BL/6 and 129S8 epithelia were infected with media control or X31 at a MOI of 0.1, for 6, 24 and 48 hours. cDNA from the cultures were analysed for CXCL1. TNF-α, IL-6 and CXCL2. Significance was assessed by unpaired t-test where * represents X31 infected:corresponding media ctrl, and ^{o} represents C57BL/6:129S8. * P<0.05, ** P <0.01, *** P< 0.001. Graphs show mean ± SEM. Data representative of two independent experiments, n=3.

4.3.3 129S8 epithelia have higher resting levels of death receptors

In the lungs of 129 mice high type I IFN production *in vivo* also correlated with increased DR5 expression on the epithelia, and its corresponding ligand TRAIL on inflammatory monocytes, thus increasing lung tissue damage in comparison to the resistant C57BL/6. This increase in lung tissue damage also associated with increased morbidity and mortality. To investigate if this increase in DR5 expression on 129 epithelia also recapitulated *in vitro,* DR5 expression was assessed on C57BL/6 and 129S8 epithelium after IAV infection (Figure 23). There was no significant DR5 induction in epithelia of either genotype after influenza infection. There is an increase however by C57BL/6 epithelia at 24 h.p.i, which correlates with the peak in matrix levels (Figure 21B). Interestingly, there is a significant increase in resting levels expression of DR5 on 129S8 epithelium. The opposite is true for the ligand TRAIL, where 129S8 have lower resting levels compared to C57BL/6. Both genotypes induce TRAIL expression following IAV infection, except for 129S8 epithelia at 24 h.p.i, where there appears to be a decrease in expression.

There is also an increase in resting state levels of FAS, another apoptosis inducing receptor, in 129S8 epithelia. Following infection, both genotypes generally have unchanged levels of FAS, except for a downregulation at 24 h.p.i in 129S8 epithelia, and 48 h.p.i. in C57BL/6 epithelia. FASL, the ligand for FAS, is induced by C57BL/6 at 6 h.p.i. only, whereas FASL expression is reduced at 6 h.p.i and unchanged at the other two time points for 129S8. The trend for TRAIL is also seen for FASL, with the resting expression levels lower in 129S8 epithelia compared to C57BL/6.

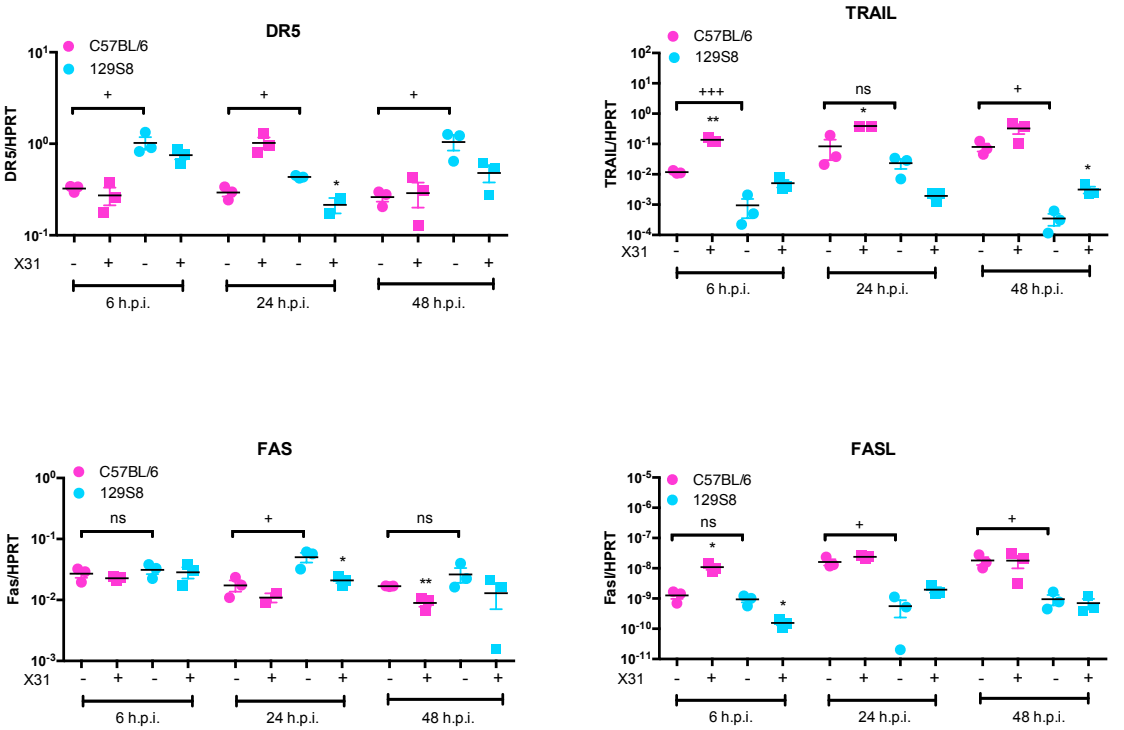


Figure 23 129S8 epithelia express higher apoptosis related receptors at resting state

C57BL/6 and 129S8 epithelia were infected with X31 at a MOI of 0.1, or media control for 6, 24 and 48 hours. cDNA from the cultures were analysed for DR5, TRAIL, FAS and FASL. Significance was assessed by unpaired t-test where * represents X31 infected:corresponding media ctrl, and $^{+}$ represents C57BL/6 media ctrl:129S8 media ctrl. * P<0.05, ** P <0.01. Graphs show mean ± SEM. Data representative of two independent experiments, n=3.

Since DR5 expression was not induced in either genotype, while *in vivo* DR5 expression increased in an IFNαβ-dependent manner (Davidson *et al.* 2014), it was considered that MTEC cultures may not respond to influenza in the same manner as epithelia in lower airway tracts or in alveoli do, since they are obtained from the trachea of mice. As primary cultures of lower airway epithelia cells are difficult to grow and maintain, we used the human lung carcinoma epithelia-like cell line, A549. This cell line represents alveolar epithelia, which can become infected in severe influenza infections. Upon infection, the human apoptosis-inducing TRAIL receptors DR5 and DR4 (TRAIL-R2 and TRAIL-R1, respectively), along with TRAIL, are significantly induced after 24 and 48 hours (Figure 24). FAS is also significantly induced, whereas its ligand FASL is only significantly increased 24 h.p.i, with a reduction in levels 48 h.p.i. This corroborates the DR5 induction on epithelia by IAV infection, in which DR5 binding to TRAIL can result in apoptosis resulting in increased damage to the lung.

Although this induction of DR5 is not seen within the 129S8 epithelium, the higher resting levels of apoptosis receptors suggest an apoptosis prone state of the 129S8 epithelium, and lack of induction following IAV infection may be due to lack of sufficiently high levels of IFN present in the cultures. However, the lack of DR5 up regulation by C57BL/6 which have previously been shown to induce high amounts of type I IFN (Figure 20) points to another contributing factor that is missing within the cultures. Future co-culture experiments could address this problem or supplementing the culture with the broncoalveolar lavage (BAL) fluid from infected 129S8 mice.

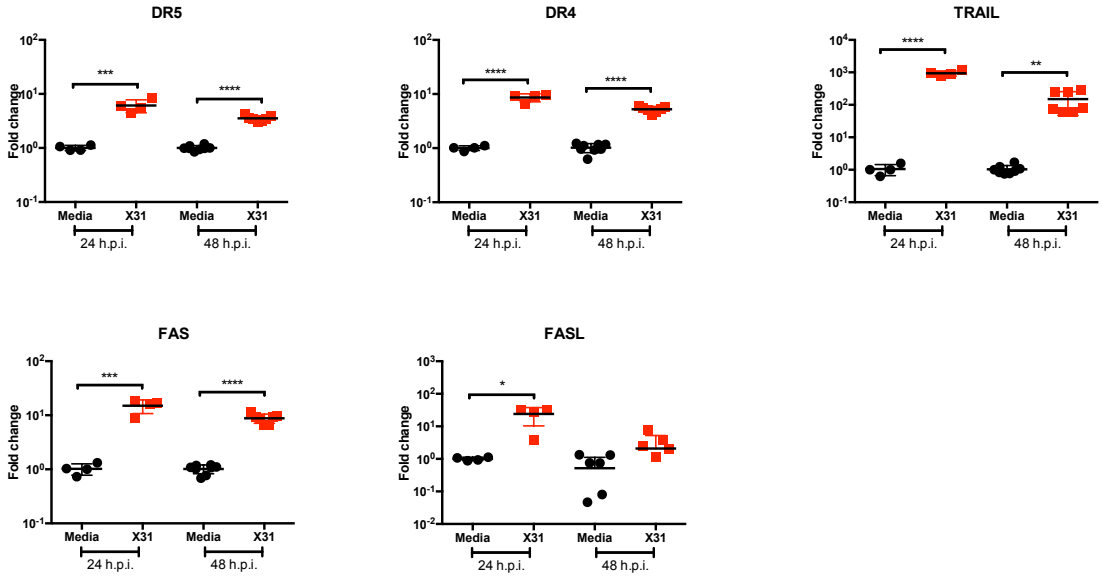


Figure 24 IAV infection induces the expression of death receptors and ligands on a human epithelial cell line

A549 cells were infected with X31 or media control and assessed for expression of TRAIL receptors 2 and 1 (DR5 and DR4, respectively), TRAIL, FAS and FASL by qPCR at specified time points. Graphs show mean ± SEM. Significance was assessed by unpaired t-test where * P<0.05, ** P <0.01, *** P<0.001, **** P<0.0001. Data pooled from two independent experiments, n=3-8.

4.3.4 129S8 epithelia are morphologically different to C57BL/6 epithelia, with reduced potential for repair

Interestingly, 129S8 MTEC cultures often fail to grow. Since the setting up of these mTEC cultures can be seen as a model of epithelial damage, in which to reach confluence again the epithelia must 'repair' by proliferating and re-creating tight junctions, we hypothesised that 129S8 epithelia may not repair as efficiently as C57BL/6. This could be due to a reduced frequency or proliferative capacity of basal cells. In addition, differentiation of basal cells may be altered, leading to epithelium remodelling as seen with ciliopathies and with goblet cell metaplasia that are associated with COPD and other chronic inflammatory lung conditions.

To establish if the epithelia of 129S8 and C57BL/6 are morphologically similar, a flow cytometry protocol of cell surface markers to stain basal, ciliated and secretory cells was designed. Since most cell markers for basal cells: p63 and KRT5 (Rock *et al.* 2009), ciliated: FoxJ1 (You *et al.* 2003), Ccno (Wallmeier *et al.* 2014), Mcidas (Boon *et al.* 2014) and goblet cells: Scgb1a1 (Zheng *et al.* 2014) are intracellular or secreted, their quantification by flow cytometry is difficult. A set of cell surface markers were used to quantify the different cell subsets: nerve growth factor receptor (NGFR) (Rock *et al.* 2009), integrin alpha 6 (CD49f) (Guo *et al.* 2012; Höfner *et al.* 2015), ceacam1a (CD66a) (Nikolai Belyaev, GSK, personal communication), prominin-1 (CD133) (Nikolai Belyaev, GSK, personal communication). NGFR, CD49f double positive, CD133 and CD66a negative were classified as basal cells (Figure 25A-gate 1). NGFR and CD49f negative, CD133 positive were classified as ciliated cells (Figure 25A-gate 2), with CD66a positive as goblet cells (Figure 25A-gate 3). NGFR, CD49f, CD133 and CD66a negative cells were classified as undifferentiated cells (Figure 25A-gate 4). An unexpected

population of NGFR negative, CD49f positive, CD133 and CD66a double-negative existed, which could be classified as a type of progenitor cell, which has not yet differentiated into a basal cell, however this has not been confirmed by PCR (Figure 25A-gate 6). Mixed populations where cells may be in transition between differentiation states also exist (Figure 25A-5). The total percentage of the different subsets is also displayed (Figure 25B). To confirm this classification of the different cell subsets is indeed correct, these different subsets were sorted and analysed for the different cell lineage markers known to be associated to them. The subset of cells classified as basal cells do express levels of the Krt5 protein and Trp63, a transcription factor associated with basal cells (Rock *et al.* 2009), more highly than any other population (Figure 25C). This confirms that the gating strategy based on NGFR and CD49f identifies a population highly enriched for basal cells. The population classified as ciliated cells did express more Ccno, Mcidas and Ccdc67 than any other cell type. However, FoxJ1 was also seen in the putative goblet cell fraction (Figure 25D). Next, the putative goblet cells were analysed for the goblet cell markers: Scgb1a1, Muc5b and Muc5ac. These were mostly expressed in the putative ciliated fraction (Figure 25E). This indicates that CD133 and CD66a are not able to distinguish between ciliated and goblet cells and are expressed by both populations, thus identifying a mixed population. Subsections 2 and 3 were combined and classified as differentiated cells, comprising both ciliated and secretory cells. It is reassuring that the undifferentiated subset expressed little no to none of the basal, ciliated or goblet cell markers, thus confirming these are in fact an undifferentiated subset of cells, and not basal cells.

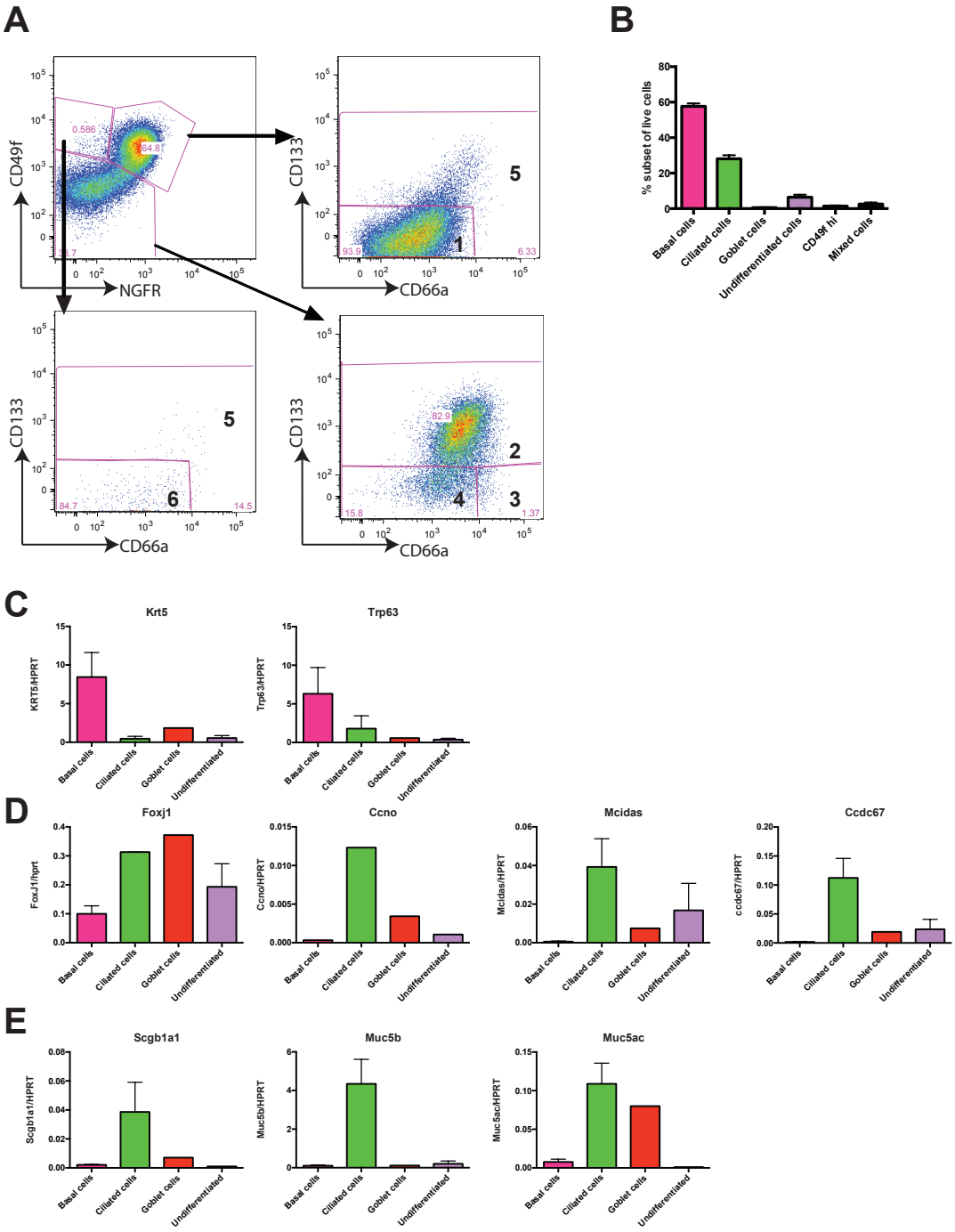


Figure 25 Determining epithelial cell sub populations

Gating strategy by flow cytometry for basal cells (1), ciliated cells (2), goblet cells (3), undifferentiated (4), mixed cells (5) and CD49fhi population (6) in C57BL/6 AECs (A). Percentage subset of live cells (B). Flow cytometry sorting of these different cell populations allowed for cDNA generation of each cell subset, which was quantified for basal cell markers: Krt5 and Trp63 (C), ciliated cell markers: FoxJ1, Ccno, Mcidas and Ccdc67 (D), and goblet cell markers: Scgb1a1, Muc5b and Muc5ac (E). Data representative of five independent experiments (A, B), n=5 (B). Data pooled from two independent experiments. Basal cells n=2-3, ciliated cells n=2-3, goblet cells n=1, undifferentiated cells n=3. CCNO n=1 for all subsets.

Using this validated flow cytometric analysis panel to analyse the different subsets of epithelial cell populations, 129S8 epithelia display drastically different amounts of the different cell subsets. When compared to C57BL/6 AECs, 129S8 AECs displayed a significantly higher percentage of undifferentiated cells and the unusual CD49fhi population (Figure 26A). They also had significantly lower basal cell and differentiated cell subsets, confirming the earlier hypothesis of a reduction of basal cells in 129 cultures. Since this flow cytometry antibody panel cannot distinguish which differentiated cell 129S8 epithelia are lacking, β-tubulin IV staining was performed directly on the mTEC cultures of both genotypes. Tubulin is the major constituent of microtubules and thus this antibody allows us to quantify ciliated cells. Intriguingly, 129S8 epithelia show significantly lower β-tubulin IV staining (Figure 26B, C). C57BL/6 AECs have many multiciliated cells whereas 129S8 epithelia have far fewer multiciliated cells and instead show a tendency for a single cilium per cell if at all (Figure 26B). This confirms that 129S8 have a phenotypically different epithelium to C57BL/6 when grown *in vitro*. If one argues that mTEC cultures model repair post injury (i.e. growth to confluence after digestion), these results could be interpreted as 129 epithelia reform differently to C57BL/6 epithelia in response to damage and as such display characteristics reminiscent of a diseased remodelled epithelium as seen in patients with ciliopathies. Mucins and goblet cell staining or PCR assays for mucin genes are needed to determine if there is also a lack of goblet cells within the 129S8 epithelium, or goblet metaplasia, as has been identified in some lung diseases. However, the whole trachea from each of these mice was removed and stained for β-tubulin IV and club cell secretory protein (CCSP). Here we do not find differences between the different hosts in the amount of ciliated or goblet cells. This therefore

illustrates that the effect we see *in vitro* may be brought about through the regeneration process.

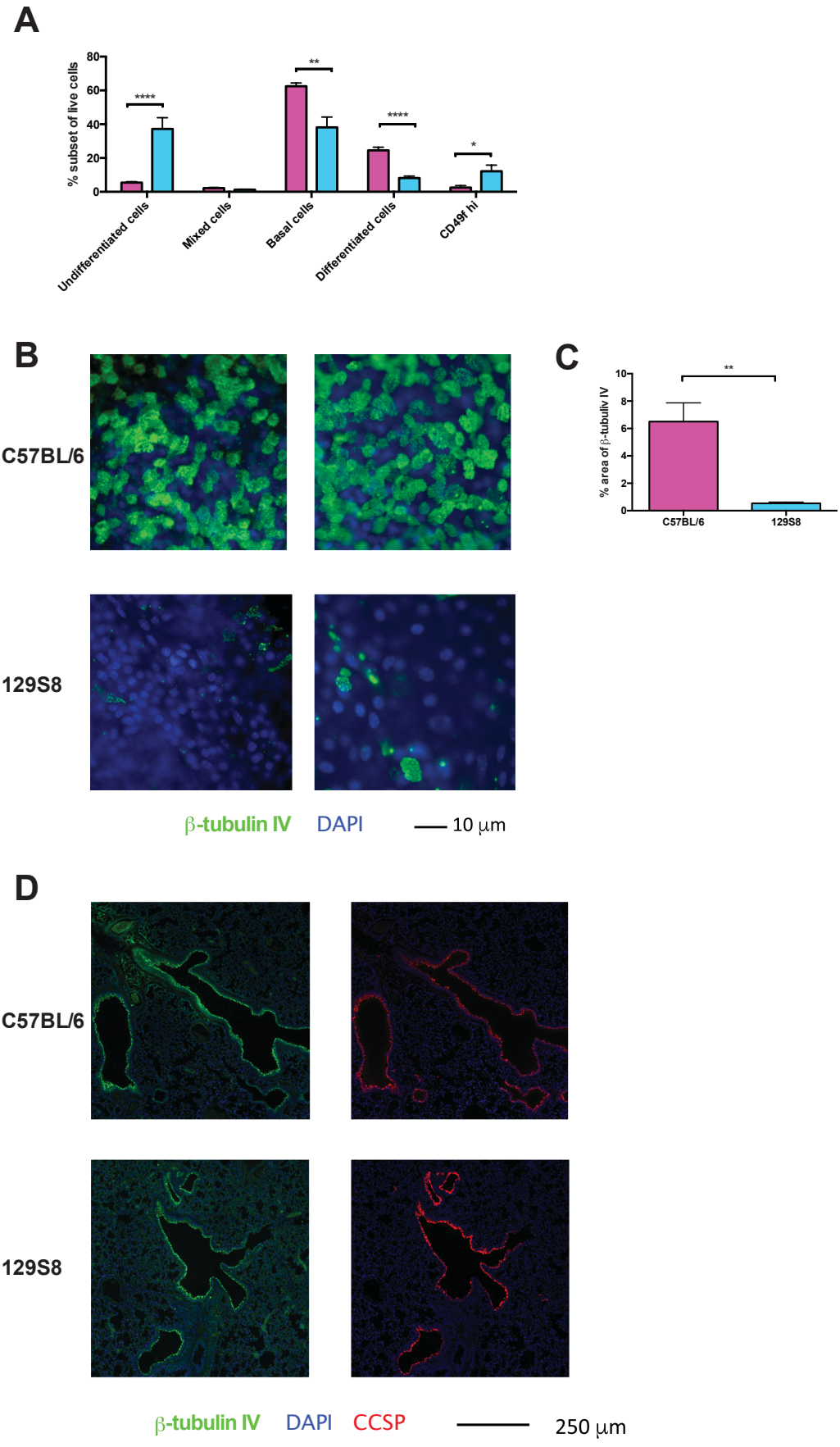

A
% subset of live cells
80
60
40
20
0
****
**
****
*
Undifferentiated cells
Mixed cells
Basal cells
Differentiated cells
CD49f hi
B
C57BL/6
129S8
β-tubulin IV
DAPI
10 μm
C
% area of β-tubulin IV
10
8
6
4
2
0
**
C57BL/6
129S8
D
C57BL/6
129S8
β-tubulin IV
DAPI
CCSP
250 μm

Figure 26 129S8 and C57BL/6 AECs are phenotypically different, with 129S8 AECs displaying a lack of cilia

Quantification of undifferentiated cells, mixed cells, basal cells, differentiated cells and CD49fhi cells by flow cytometry in C57BL/6 and 129S8 AECs (A). Immunofluorescence of C57BL/6 and 129S8 AECs for β-tubulin IV and DAPI (B). Quantification of percentage area of β-tubulin staining of C57BL/6 and 129S8 AECs (C). Whole tracheas from C57BL/6 and 129S8 mice were stained for β-tubulin IV, DAPI and CCSP (D). Significance was assessed by unpaired t-test where * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Data pooled from five independent experiments, n=4-17.

We hypothesised that 129S8 epithelium may have a repair defect which may explain the increased morbidity and mortality after IAV infection. To determine if this was true, fully-grown cultures were scratched and the transepithelial electrical resistance (TEER) was recorded to determine the time at which the cultures reach confluence again (R>1000 $m\Omega/cm^2$). Scratching the cultures induces damage which they need to repair to re-establish confluence. The cultures were also re-scratched until they could no longer repair the scratch (Figure 27A). Upon the first scratch there was no significant difference between the two genotypes in the time taken to reach confluence (Figure 27B), however around 20% of 129S8 cultures were unable repair the scratch and reach confluence again (Figure 27C). Upon the second and third scratch, 129S8 epithelia have a significantly reduced ability to reach confluence. 129S8 take longer to repair the scratch, with 20 - 60% of the cultures failing to repair the scratch at all (Figure 27B, C). To further confirm that 129S8 epithelia have reduced ability to repair, EdU was added to un-scratched and scratched cultures and analysed after 24 and 48 hours. At both time points 129S8 AECs had fewer EdU positive cells than C57BL/6 AECs in the unscratched cultures (Figure 27D). Upon scratching the cultures, the percentage of EdU positive cells do not increase after 24 hours post scratch (h.p.s.), but the difference between the two genotypes is still evident. 48 h.p.s EdU positivity does increase in both genotypes however the levels in 129S8 AECs do not reach those of C57BL/6 AECs. However, the extent of a scratch injury can vary between transwells, and future methods that will introduce a uniform level of injury to a transwell should be used.

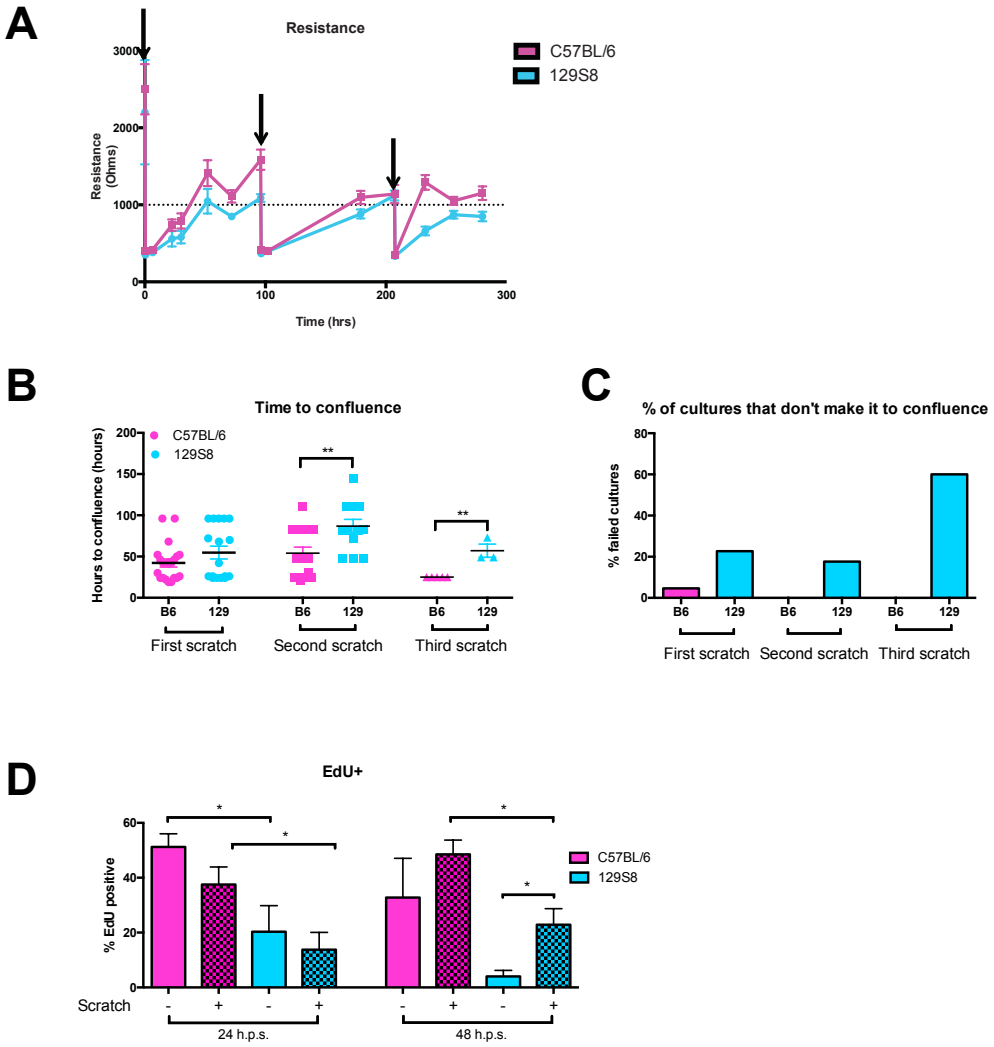


Figure 27 129 epithelia undergo less proliferation and delayed repair after mechanical injury in comparison to C57BL/6 epithelia

C57BL/6 and 129S8 AEC cultures were scratched and re-scratched with the transepithelial resistance measured until confluence is reached (R>1000 mΩ/cm^2) (A). The time taken to reach confluence (B) and percentage of cultures that don't reach confluence is graphed (C). Un-scratched and scratched cultures were analysed for EdU+ cells by flow cytometry 24 and 48 hours post scratch (h.p.s). Graphs show mean ± SEM. Arrowheads indicate when the AEC cultures where scratched. Significance was assessed by unpaired t-test where * P<0.05, ** P <0.01. Data representative of seven independent experiments, n=3 (A). Data pooled from seven independent experiments, n=3-21 (B, C). Data representative of three independent experiments, n=3-4 (D).

To determine if this lack of repair is recapitulated *in vivo*, C57BL/6 and 129S8 mice were infected with X31, and EdU positivity of lung cells was quantified. Pre-determination of lung damage of these mice following IAV infection and before EdU administration could not be performed without first removing the lung for staining with damage markers such as TUNEL, which stains apoptotic cells. Due to the higher susceptibility of 129S8 mice to IAV-induced disease, the IAV doses administered to C57BL/6 and 129S8 mice were modified to levels previously found within the lab to induce similar weight loss. Thus, C57BL/6 mice were infected with 2.4 $x10^4$ $TCID_{50}$, whereas 129S8 mice were infected with 800 $TICD_{50}$. Weight loss is one clinical symptom of IAV disease, however, it is does not conclusively indicate the levels of damage induced.

There is no significant difference between 129S8 and C57BL/6 EdU positivity levels be it in their hematopoietic or non-hematopoietic cells (Figure 28A, B). Although infected with less IAV, 129S8 mice had increased weight loss and showed no signs of recovering like the C57BL/6 mice (Figure 28C). This divergent response to IAV infection demonstrates how difficult it is to normalise for damage using IAV as the damage inducer. As it is not possible to assess lung damage in the individual mice used for EdU treatment, this type of experiment was not further pursued. Future *in vivo* experiments could implement another method of inducing lung damage such as naphthalene, to measure damage and repair capabilities between these mice strains.

Overall, it is clearly evident that 129S8 epithelia are not only more responsive to IFN exposure and IAV infection but have a reduced potential for repair and show impairments in basal cell numbers and epithelial cell differentiation reminiscent of epithelia remodelling in chronic inflammatory diseases. These

results demonstrate that genetic changes can impact the biology of airway epithelia resulting in different epithelial responses and regeneration. In particular, the epithelial differences observed in 129S8 epithelia are consistent with hypercytokinemia and decreased repair of the lung following IAV infection, thus potentially contributing to morbidity and mortality.

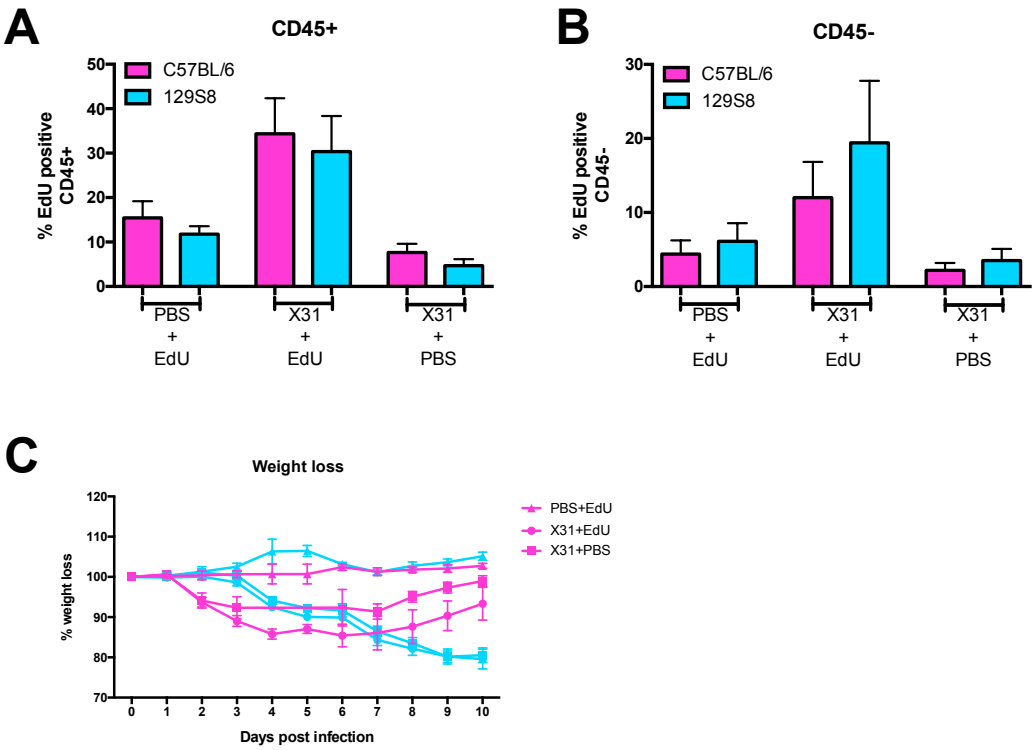


Figure 28 Proliferation of cells following IAV-induced damage does not differ between C57BL/6 and 129S8 mice

C57BL/6 and 129S8 mice were infected with X31 (2.4 x 10^4 $TCID_{50}$ and 800 $TCID_{50}$ respectively) and treated with EdU on days 6, 7, 8 and 9 post infection. Whole lungs were taken and analysed for EdU positivity in CD45+ (hematopoietic cells) (A) and CD45- cells (non-hematopoietic cells) (B) 10 d.p.i. by flow cytometry. The weights of the mice were recorded throughout infection (pink: C57BL/6, blue: 129S8) (C). Graphs show mean ± SEM. Data pooled from four independent experiments, n=9-10 (A, B). Data representative of four independent experiments, n=3 (C).

4.4 Conclusions and Discussion

Severe influenza infection correlates with rapid development of lung injury and a vigorous inflammatory response (Cate 1987; Kaufman *et al.* 2009; Mauad *et al.* 2010). However, individuals differ in their response to infection, which may be due in part to differences in host genetics (Peiris *et al.* 2009). We hypothesized that the airway epithelia may play a role in the divergent response to IAV. We have shown that AECs generated from an IAV susceptible and a resistant in-bred mouse strains diverge in the response to IAV, in the production of IFNs, ISGs, and proinflammatory cytokines and chemokines. The composition and regenerative capabilities also differ between these two host strains. 129S8 AECs have a reduced ability to repair compared to the resistant C57BL/6 AECs. When 129S8 AECs do repair, we see an epithelial phenotype that is drastically different to C57BL/6 epithelia – fewer basal cells, and fewer differentiated cells. We therefore believe that genetic differences can determine the response of the epithelia to IAV infection, and contribute to the ability to repair lung tissue injury. This change in the ability to respond to infection and repair may contribute to the increased morbidly and mortality following IAV infection *in vivo*.

IAV preferentially infects and replicates within the airway epithelium, which responds by inducing the antiviral cytokines type I and III IFNs. Although historically type I IFNs were shown to be protective during IAV infection, studies have shown that they can be pathogenic in high quantities as in the 129 mice (Boon *et al.* 2009; Davidson *et al.* 2014). We therefore postulated that this *in vivo* phenotype would be recapitulated *in vitro*, with a higher production of IFNs by the 129S8 AECs in comparison to the C57BL/6 AECs. However, 129S8 AECs were not found to be high IFN producers but were in fact scarcely inducing IFNs at all. This lower

amount of IFNs at the level of AECs may be detrimental in itself for these cultures. This contrasts the *in vivo* findings, but this may be due to lack of the IFN producing innate immune cells in the system, such as pDCs, which have been described to induce robust amounts of type I IFN and be recruited in large numbers to the lung following influenza infection. In fact, depletion of PDCA-1+ cells protects 129 mice from severe influenza (Davidson *et al.* 2014). It would therefore be interesting to quantify IFN production from a co-culture of AECs and pDCs and/or other innate immune cells.

The *in vivo* results only describe severity correlating with a high type I IFN response. In our system type III IFN, a second totally independent, yet redundant IFN system, which acts exclusively on epithelial cells (Kotenko *et al.* 2002; Onoguchi *et al.* 2007; Crotta *et al.* 2013) is the most upregulated IFN in both 129S8 and C57BL/6 AECs. This could indicate that type III IFN may play a bigger role during influenza infection in AEC cultures as compared to the situation as a whole lung.

We did not find the high IFN response by the 129S8 epithelia as is observed *in vivo* in the whole lung. However, the greatest peak of IFN induction *in vivo* is 2/3 d.p.i. (Davidson *et al.* 2014). It would therefore be interesting to repeat this experiment at later time points. Unfortunately, the cytolytic effect of IAV could potentially kill the entire culture and therefore, the results may not fully recapitulate what is observed *in vivo*. Intriguingly, a study pointed to endothelial cells, a cell type only infected in severe IAV, as orchestrating the excessive cytokine response to influenza infection (Teijaro *et al.* 2011). This would suggest that it is not the epithelia from the proximal lung that are inducing the excessive IFNs in severe IAV but rather another cell type, be it stromal or non-stromal. However, to see so little

IFN induction is surprising and may therefore be due to the remodelling of the epithelium after injury which will be discussed at length later in this chapter.

In vivo results from the lab have shown that bone marrow chimeras of 129 mice reconstituted with type I IFN knock out bone marrow have low IFN levels and yet are more susceptible to IAV infection. Therefore, the production of IFN may not be the limiting factor, but rather the responsiveness of the cells to it. Severe IAV infection correlates with a 'cytokine storm' leading to excessive lung damage and ultimately death of the host (de Jong *et al.* 2006; Taubenberger and Morens 2006; Vigneswaran *et al.* 2007; Zeng *et al.* 2007; Peiris *et al.* 2009; Stary *et al.* 2009; Bernardo *et al.* 2013). This lead us to hypothesize that 129S8 AECs would be more responsive to IAV by producing more ISGs, proinflammatory cytokines and chemokines, and apoptosis related genes than the resistant counterpart strain. We found that this was somewhat the case with 129S8 AECs producing more ISGs, and proinflammatory cytokines and chemokines. However, we did not find any induction of death receptors on the AECs in either genotype after IAV infection.

This lack of death receptor induction in the 129S8 AECs may be due to the absence of type I IFNs which have previously been shown to induce DR5 on the lung epithelia (Herold *et al.* 2008; Davidson *et al.* 2014; Brauer and Chen 2016; Hui *et al.* 2016). Previous findings within the lab have shown that 129 mice have higher numbers of apoptotic epithelial cells following IAV infection due to the higher IFNαβ production (Davidson *et al.* 2014). However, if type I IFN alone is required to induce DR5 on epithelia, we should see an induction in the C57BL/6 AECs, where we do not. We do however find death related apoptosis genes significantly induced in a different culture system: A549 cells, following IAV infection. This suggests that another contributing factor may be missing from the mTEC cultures, such as innate

immune cells or type I IFN indirectly causing DR5 expression through the induction of cytokines that are not present in these cultures. Therefore incorporating innate immune cells into the culture could induce the needed IFN and/or other cytokines leading to up regulation of DR5 on the epithelia. BAL fluid collected from IAV infected 129S8 mice would provide another way to add the missing recruited immune cells and cytokines and chemokines that would normally be acting on the AECs. Utilizing the two genotypes again in these experiments could further delineate the genetic contribution to severity of disease. Apoptosis is not only induced by DR5 expression, and therefore TUNEL staining of the epithelium could be carried out to fully decipher the epithelium integrity before and after IAV infection. Nevertheless, the higher resting state level of death receptors on 129S8 AECs compared to C57BL/6 AECs is interesting and suggests a possible apoptosis prone state of the epithelium.

We have shown that 129S8 epithelia do not recapitulate the high type I IFN phenotype as observed *in vivo*. However, they are more responsive to infection and IFNs in the production of ISGs, and proinflammatory cytokines and chemokines. These responses from the 129S8 AECs are not massive and do not fully reflect the 'cytokine storm' observed *in vivo*.

A hallmark of infection by respiratory viruses is productive infection of and the subsequent destruction of the airway epithelium. Following resolution of the infection, this airway damage must be repaired to allow for total recovery of the host. Digesting and plating out the AECs is a form of damage and repair model without the complication of an active influenza infection, thus allowing us to easily decipher any differences following injury between the phenotype of 129S8 versus C57BL/6 AECs. We hypothesized that the 129S8 AECs would be less capable of

repairing. We confirmed this hypothesis, but we also discovered that the 129S8 epithelium has a remodeled phenotype following injury showing less basal and differentiated cells.

To quantify the different cell types of the AECs we had to set up an antibody flow cytometry panel. The upper airway epithelium is made up of three main cell types: basal cells, ciliated cells and secretory cells. Although NGFR and CD49f are well known as basal cells markers (Rock *et al.* 2009; Guo *et al.* 2012), there are no known cell surfaces markers for ciliated or secretory cells, making flow cytometric analysis of these cells difficult. Our antibody panel employed here is an attempt to use cell surface markers to quantify these cell types. However, as of yet it is not fully known what the CD49fhi single positive population is, as they were not easily sortable due to their lower number in C57BL/6 cultures. Sorting of 129S8 AECs may allow for sorting of this population and quantifying for the expression of known markers of the different cell subsets. However, we found it difficult to obtain sufficient cell numbers from this sorting process for downstream analysis. Comparing the different cell subsets it was surprising to find significant differences between the two genotypes. 129S8 AECs displayed more undifferentiated and CD49fhi single positive cells, and less basal and differentiated cells than in the C57BL/6 AECs. The increased undifferentiated and CD49fhi single positive cells, and the lower amount of basal cells could suggest that there may be a reduced ability of basal cells to proliferate. The lower percentage of differentiated cells also suggests a block in differentiation.

Interestingly, in a study comparing B6 to 129 responses to IAV, basic fibroblast growth factor (bFGF), a growth factor important for epithelial repair, was found to be the only cytokine downregulated in 129 mice compared to the B6 mice

(Zhou *et al.* 2016). Other known proliferation factors such as epidermal growth factor (EGF), and transforming growth factor alpha (TGF-α) should also be tested for blockade within these 129S8 cultures (Aaronson *et al.* 1990; Ferriola *et al.* 1991).

The current flow cytometry panel employed here does not allow us to distinguish differentiated cells into either ciliated or secretory cells within the 129S8 culture. We therefore used immunofluorescence of the cultures with β-tubulin IV to quantify ciliated cells. Here we show a striking lack of ciliated cells within the 129S8 culture. We did not see this effect *in vivo* when the whole trachea was stained, however such phenotypes may be stronger *in vitro* than *in vivo*, and more subtle effects may occur *in vivo*. This may also tell us that this effect only occurs when damage and repair is initiated.

A normal ciliated cell should display many cilia on its surface, whereas in most cases the 129S8 epithelia show rather a trend for one or few cilia. To fully see if there is a defect in multiciliogenesis electron microscopy should be carried out. This could nonetheless point towards a blockade in the downstream mechanism for the formation of cilia. For the formation of cilia, MCIDAS and CCNO must be expressed for basal cells to exit the cell cycle and allow for centriole amplification. CCNO and FOXJ1 induce apical docking of the centrioles and FOXJ1 induces the expression of motile ciliate proteins (Boon *et al.* 2014). Notch functions as a repressor of MCIDAS and could potentially be overexpressed in regenerating 129S8 epithelium. The aryl hydrocarbon receptor (AhR) is downstream of Notch signalling and has been shown to not only be required for detoxification but also for the generation of multiciliogenesis, as blockade of AhR resulted in reduced cilia in mTEC cultures (Villa *et al.* 2016). Interestingly, 129 mice are AhR hypomorphs and

thus this may account for the reduction in ciliated cells. Overall, any mutations or blockade of MCIDAS, CCNO, FOXJ1 or AhR could result in reduced generation of cilia; therefore they may be interesting factors to quantify within the 129S8 epithelium.

Since the 129S8 often do not grow when plated, we hypothesized that they would have a decreased ability to repair. To confirm this the cultures were injured by scratch assays repeatedly. Indeed 129S8 cultures took longer to repair the scratch and quite often failed to do so, which likely is due to the lower amount of proliferation we find. This could reflect the lower amounts of basal cells and potentially club cells within these cultures. The reduced ability of 129S8 epithelium was not recapitulated *in vivo* when mice were infected with X31 and treated with EdU. However, this system is difficult to control as a method of damage induction since the mouse strains under comparison respond differently to IAV infection. It was therefore difficult to normalize for damage as there is no reliable measure of damage inflicted on the epithelium before the EdU is administered, without first culling the mice. To fully assess the amount of damage inflicted, mice would have to be culled and the lungs stained for damage markers or lack of the aforementioned cell subsets. Influenza infection also causes many secondary effects, which could be affecting the ability of the epithelium to repair as discussed in Chapter 6.

Another method of lung epithelium damage should be carried out such as naphthalene treatment. Naphthalene is already extensively used to induce damage to the airways and quantify repair (Lawson *et al.* 2002; Carvalho-Oliveira *et al.* 2009). Naphthalene is a chemical which, when metabolized by club cells ablates them. Ciliated cells squamate and cover the basement membrane. The lung

epithelium must then repair by progenitor and basal cell proliferation, which starts 24 hours after injury. Differentiation occurs allowing for full restoration of the epithelium and function of the lung, which can take up to several weeks (Carvalho-Oliveira *et al.* 2009). Lawson *et al.* used this method to quantify repair in C57BL/6 and 129/SvEv mice and showed that 129SvEv had a delay in the formation of cilia, reduced cell density, and proliferated for longer compared to C57BL/6 mice (Lawson *et al.* 2002). This is highly encouraging and somewhat confirms our own findings. We plan to employ this method in future studies

Overall, it is evident that genetic differences of the airway epithelium can determine the outcome of disease. Epithelia from the IAV-susceptible mouse strain displayed an increased responsiveness to IFNs and IAV infection, remodeling of the epithelium and reduced potential for repair after injury. This reduced potential for repair and changes in the different cell subsets is interesting as it reflects what is observed in chronic lung illnesses. This may highlight that there may be a genetic contribution to the generation of chronic lung illnesses where repeated lung insult and injury leads to remodeling of the epithelia in some individuals.

Chapter 5. Therapeutic potential of IFNs during IAV infection

Work in this Chapter was undertaken with Dr Sophia Davidson as a joint project. Where indicated, experiments were carried out co-operatively. Much of the experimental results in this chapter have been published: 'IFNλ is a Potent Anti-Influenza Therapeutic without the Inflammatory Side Effects of IFNα Treatment' Sophia Davidson[1], Teresa M McCabe[1], Stefania Crotta, Hans Henrik Gad, Edith M Hessel, Soren Beinke, Rune Hartmann and Andreas Wack. EMBO Mol. Med. DOI 10.15252/emmm.201606413 (2016). [1]Joint first authors

5.1 Background

IAV can cause up to five million cases of severe illness and up to 500,000 deaths annually (Krammer *et al.* 2015). IAV can also cause pandemics, such as the 1918 "Spanish Flu" in which an estimated 50 million people died globally (Johnson and Mueller 2002). At present, there exists the constant threat of highly pathogenic H5N1 and H7N1 avian influenza strains, which demonstrate the burden of IAV and the need to better protect from and treat IAV infections.

Currently, the best method for protection from IAV is immunization with inactivated or live attenuated vaccines. However, existing vaccines rarely induce broadly neutralizing antibodies, thus they cannot provide protection against heterologous IAV strains (Krammer *et al.* 2015). Therefore, each year new vaccines must be made to match the current circulating strain in addition to

predicting viruses that are the most likely to circulate in the upcoming influenza season. This has a number of potential problems. Many influenza virus strain candidates grow poorly in eggs making it difficult to obtain the vaccine. Vaccines require six months to prepare, thus they must be made well before the upcoming influenza season. Incorrect predictions of which strains are likely to circulate could potentially be made, thus rendering the vaccines partially inefficient. Furthermore, some IAV strains do not appear until late in the season and as such may be missed when the vaccine candidates are chosen. Due to the long time frame for creating a vaccine, creating an additional vaccine late in the season is impractical.

Once infected with IAV, the main treatment options are antivirals. The prophylactic efficiency of antivirals varies between 80 to 90%, and ideally need to be administered within 48 hours of the onset of symptoms to reduce severe complications and deaths (Davies *et al.* 1964; Younkin *et al.* 1983; Reuman *et al.* 1989; Spagnuolo *et al.* 2016). There are two classes of antivirals: ion channel blockers (amantadine and rimantadine), and inhibitors of influenza neuraminidase (oseltamivir, zanamivir, peramivir and laninamivir), which act directly on viral proteins (Jefferson *et al.* 2006). However, emergence of drug resistance, due to the high natural mutation rate of IAV, has been observed. In fact since 1994, reports have described adamantine-resistant A (H3N2) (Bright *et al.* 2005), rimantadine-resistant strains of influenza A virus (H3N2) (Hayden *et al.* 1989), oseltamivir-resistant seasonal A (H1N1) viruses and adamantine-resistant pandemic A(H1N1) (Pizzorno *et al.* 2011). This resistance has also been found to be transmissible, which is worrying as they may widely replace susceptible strains. Thus there is a need to target novel IAV and strains that cannot be vaccinated against, without driving drug resistance. Antiviral host factors would provide ideal treatment options,

since they avoid the issue of drug resistance. Type I and III IFNs could represent one such family of immune activated antivirals.

As previously mentioned, type I IFNs are a family of cytokines which are known for their antiviral properties but are also strong immunostimulants. Binding to IFNAR results in the transcription of hundreds of ISGs (Randall and Goodbourn 2008). Importantly, two of these ISGs have been known to be significant restriction factors of IAV: IFN regulator factor 7 (IRF7), and orthomyxovirus resistance gene (Mx). IRF7 is essential for the amplification of type I and III IFNs during IAV infection (Ciancanelli *et al.* 2015). MxA, the human homologue of Mx1, can also restrict IAV replication by interfering with viral protein transport, synthesis or translocation (Pavlovic *et al.* 1995).

Type III IFNs, another more recently discovered family of IFNs, are also induced during viral infection which initiate the same JAK/STAT signalling cascade to induce the same ISGs as type I IFN. They however do not bind through the IFNαβR, but through their own heterodimeric receptor complex composed of IL-10R2 and IFNλR1 subunits. This receptor, unlike IFNαβR, is not ubiquitously expressed, but rather is expressed primarily at mucosal epithelia such as the lung and gut (Kotenko *et al.* 2002; Sheppard *et al.* 2002).

Mice deficient for both IFNαβR and IFNλR were found to be highly permissive to a number of respiratory and other viruses (Mordstein *et al.* 2008). Not only did mice deficient for both receptors display increased viral titres compared to Wt mice, but also to mice deficient for just IFNαβR or IFNλR. These increased viral titres correlated with an increase in disease burden and host mortality. The IFNαβR deficient mouse controlled the virus better than the double knock out mouse, demonstrating the redundant role of type I and III IFNs within the AECs. This was

further confirmed by Crotta *et al.* who showed that only AECs deficient for both type I and III receptors in AECs resulted in high IAV loads and as such host morbidity and mortality, even with an intact Wt hematopoietic immune response (Crotta *et al.* 2013). The lung epithelium, the first host target of IAV infection, is therefore essential in the induction of antiviral ISGs and thus the control of IAV.

IAV has evolved ways to antagonize type I and III IFN induction and signalling through the action of the viral NS1 protein (Jia *et al.* 2010). As such treatment by the exogenous addition of more of these cytokines to an infected host may serve to bypass this virus-mediated block of IFN induction. Type I IFNs have previously been suggested as a possible treatment for IAV during infection (Finter *et al.* 1991). However, type I IFNs, although they are excellent inducers of antiviral genes, also have the potential to induce immunopathology, resulting in exacerbated disease (Davidson *et al.* 2014). Type III IFNs have previously been shown to have a less adverse effect compared to type I IFNs in clinical trails for hepatitis C (Muir *et al.* 2014). Therefore its therapeutic potential in IAV is clinically relevant. However, the Davidson *et al.* study was undertaken on Wt in-bred mice which have been shown to carry nonfunctional alleles for Mx1 (Staeheli *et al.* 1988). Therefore, given the potent antiviral capability of Mx1, it could be argued that the pathogenic, rather than protective, effect of a high IFNαβ response is due to the lack of the Mx protein. Indeed pretreatment of congenic mice, which are genetically backcrossed to express a functional Mx1 protein: A2G-Mx1, with IFNα displayed complete protection from lethal 1918 H1N1 infection, whereas only partial protection was observed in non-IFN pretreated, IAV infected controls (Cilloniz *et al.* 2012). Furthermore, pretreatment of A2G-Mx1 mice with IFNα also protected mice against infection from the highly pathogenic H5N1 strain (Tumpey *et al.* 2007).

As the world's population continues to expand rapidly, and contact with natural avian IAV hosts increases, the potential for novel IAV strains to cross the species barrier also escalates. This, along with the difficulty of developing the right vaccines, the high mutation rate of IAV, and the antiviral resistance demonstrates the urgent need for a protective host-immune mediated treatment option. IFNs, a host induced family of antiviral cytokines, therefore become very interesting as a treatment option and their role as therapeutics is assessed here. Given the known potent immunomodulatory effects of type I IFNs, type III IFNs, which have not previously been assessed as a potential treatment option for IAV, are also studied here. As the IFNλR is only expressed on mucosal epithelia, the addition of IFNλ may not stimulate the hematopoietic system and therefore may not drive the immunopathology ascribed to IFNα.

5.2 Hypothesis and Aims

Severe IAV induced disease is characterised by a robust immune response and lung tissue damage. Type I (IFNα) and type III (IFNλ) IFNs are potent antivirals rapidly induced following infection. Given the vaccine difficulties, IFNα4 and IFNλ2 were used to assess their potential as viable treatment options against IAV-induced disease in B6.A2G-Mx1 mice. A viable treatment option would be one that protects the host from viral and host-induced tissue damage, does not induce immunopathology, and lowers the overall disease burden. MTEC cultures will be used to identify equivalent doses of IFNα4 and IFNλ2.

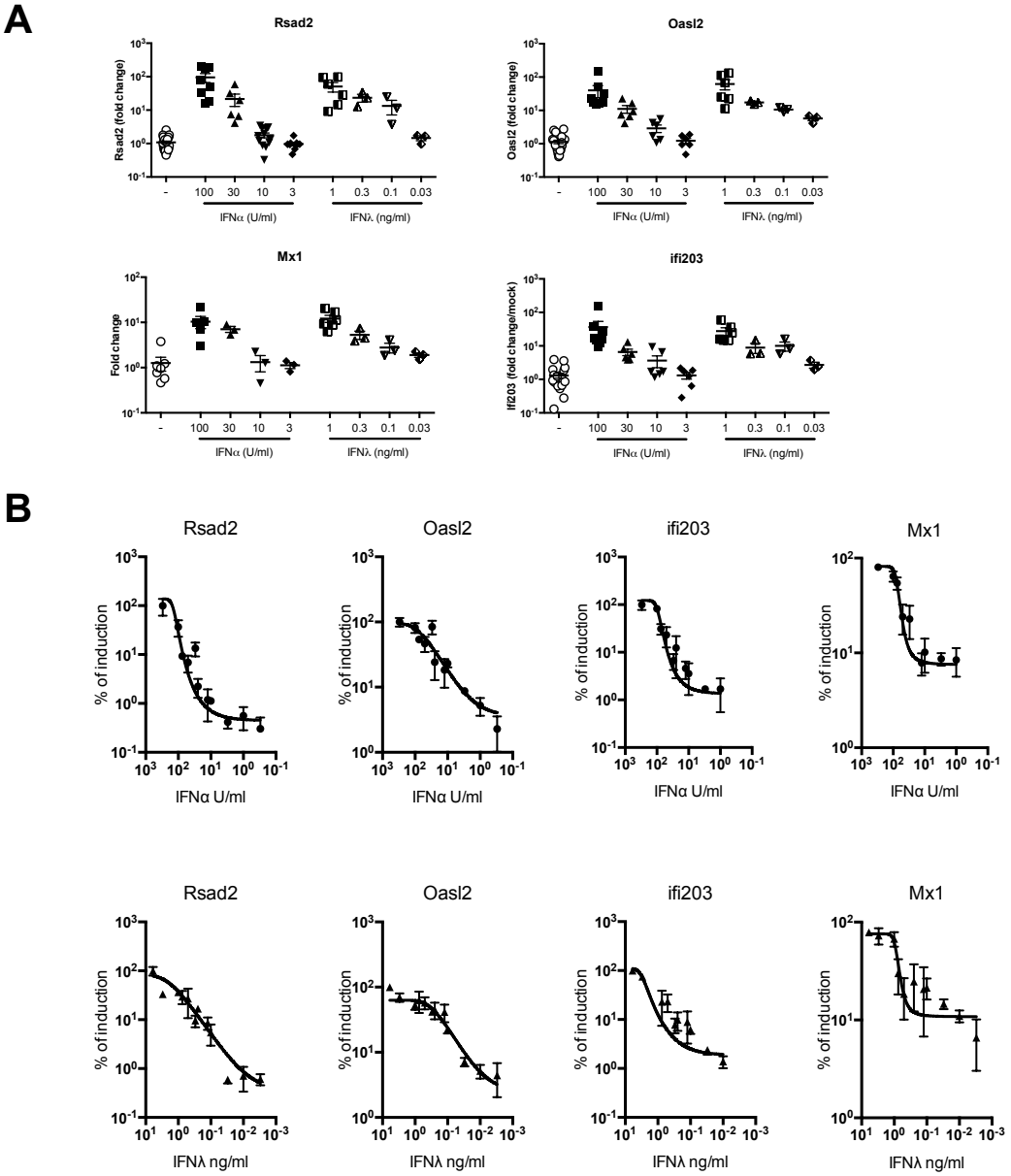
I hypothesize that:

- Due to the different distribution of type I and III receptors, IFNα4 will induce immunopathology by stimulating the innate immune cells, whereas IFNλ2 will not
- Therefore, IFNλ2 may be the better option as a therapeutic in influenza

5.3 Results

5.3.1 IFNλ and IFNα are protective if given before IAV infection, but only IFNλ is protective in an on-going infection

To ascertain equivalent doses of type I IFN, IFNα, and type III IFN, IFNλ, the induction of ISGs were assessed on AECs. AECs were treated with a serial dilution of either IFNα or IFNλ and the ISGs Rsad2, Oasl2, ifi203 and Mx1 (Figure 29A) were quantified. As expected, AECs respond to both types of IFN. The dose response curves were then used to determine EC50 values, which allowed us to calculate a conversion ratio to establish equipotency between IFNα4 and IFNλ2 (Figure 29B). The final conversion ratio of 0.558 was determined by the geometric mean of the ratios obtained for all ISGs assessed and applied to treat mice expressing the functional Mx1 gene (B6.A2g-Mx1) and of various cell types with equivalent doses of IFNα4 and IFNλ2.



$$\frac{\text{EC50 IFNα ISG induction}}{\text{EC50 IFNλ ISG induction}} = \text{Conversion Ratio}$$

Figure 29 Determination of equivalent doses of type I and III IFNs using AECs

AECs were stimulated for 4hrs with IFNα4 or IFNλ2, and induction of ISGs was assessed by qPCR. Prism 6 software was used to perform a curve fit (Sigmoidal, 4PL), generate a dose response curve and calculate a half-maximal effective concentration (EC_{50}) for each gene assessed for each treatment. A conversion ratio was then generated using the indicated formula for each ISG and a final conversion ratio was taken from the average of all ISGs assessed. Values represent means ± SEM. Data is pooled from 2 independent experiments, n=3-7.

Intranasal IFN treatment with either IFNα4 or IFNλ2 of mice prior to infection with the pathogenic IAV strain, PR8 (strain A/Puerto Rico/8/34 H1N1), protected mice from morbidity and mortality, as has been previously shown within the literature (Tumpey *et al.* 2007; Mordstein *et al.* 2008; Cilloniz *et al.* 2012) (Figure 30A). This protection correlated with undetectable viral titres within the lung at 4 days post infection, whereas the vehicle control groups displayed higher viral tires, weight loss and over 55% mortality (Davidson *et al.* 2016). This indicates that both types of IFNs can induce an antiviral environment within the lung, preventing IAV infectivity and replication.

As patients only seek medical help when they display symptoms of IAV disease, it is not feasible to pre-treat the entire population during a pandemic. Therefore intranasal IFN treatment during an on-going IAV infection represents a more realistic clinical option and as such the effectiveness of IFNα4 and IFNλ2 administered after IAV infection is assessed. B6.A2g-Mx1 mice were infected with PR8, and treated intranasally with vehicle control, IFNα and IFNλ on days 2, 4 and 5 post infection (figure 30B). Here we see a divergent outcome depending on the type of treatment. IFNλ2-treated mice were better protected against IAV-induced disease, exhibiting decreased morbidity and mortality compared to the vehicle control group. IFNα4 treatment however resulted in increased morbidity and mortality. This divergence in disease outcomes did not however correlate with a difference in viral loads. IFNα4 and IFNλ2 treatment both resulted in significantly lower viral loads compared to vehicle control treated groups (Davidson *et al.* 2016).

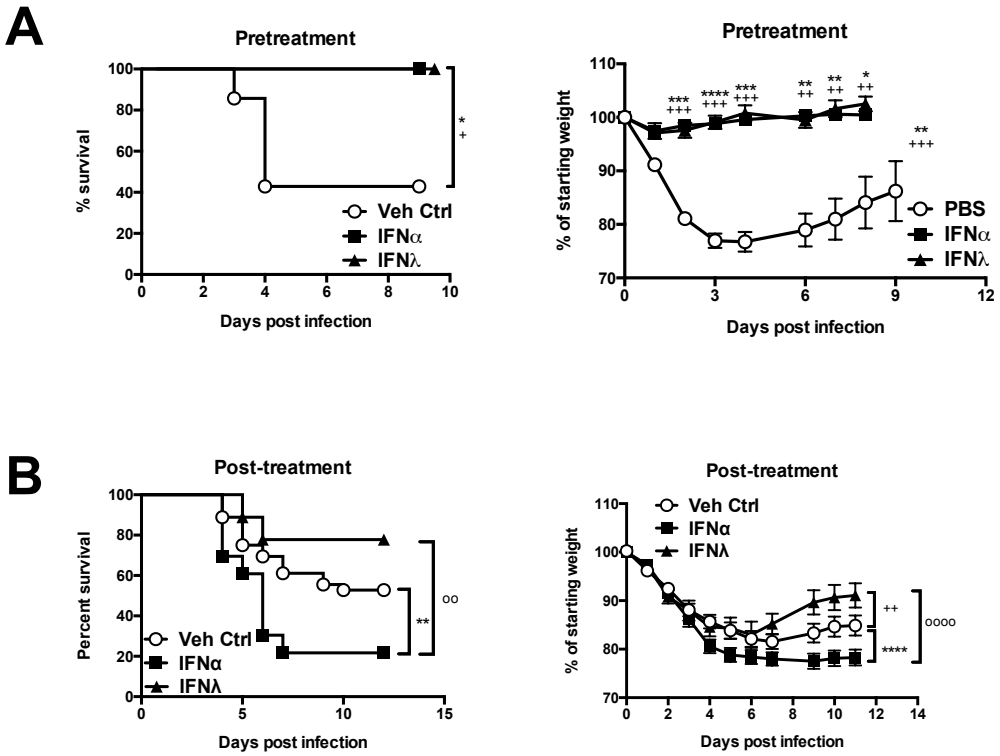


Figure 30 IFNα4 and IFNλ confer protection if given before IAV, however only IFNλ is protective during an active IAV infection

B6.A2G-Mx1 mice were pre-treated with equivalent doses of IFNα4 (1.45 μg/50ul) or IFNλ2 (2.6 μg/50ul), or Veh Ctrl (50ul) 24 hrs. prior to infection with PR8. Weight loss and survival was assed throughout infection (A). B6.A2G-Mx1 mice were infected with PR8 and treated with equivalent doses of IFNα4 or IFNλ2, or Veh Ctrl 2, 4 and 5 days post infection. Survival and weight loss was monitored throughout (B). Significance was assessed by Log-rank (Mantel-Cox) test (survival), and 2-way ANOVA (weight loss). * indicates IFNα4:Veh Ctrl, $^{+}$ indicates IFNλ2:Veh Ctrl and ^{o} indicates IFNα4:IFNλ2. Symbols to the right indicate statistical significance of the whole curve. * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001. Data is pooled from two independent experiments, n=5-7 (A). Data (n=17-34) is pooled from six independent experiments that were performed *by myself and by Dr Sophia Davidson.*

5.3.2 Overlapping and divergent effects of IFNα and IFNλ

Given the divergent outcome between IFNα and IFNλ treatment following IAV-induced disease, which does not correlate with differences in viral load, there must be another host immune aspect responsible for IFNλ protection. High IFNα levels following IAV have already been associated with immunopathology, epithelial cell damage, and mortality (Davidson *et al.* 2015). Therefore, the effect of IFNα and IFNλ treatment on innate immune cells and AECs was assessed. Bronchoalveolar lavage (BAL) fluid throughout IAV infection was collected and measured for the inflammatory cytokines: IL-6, CXCL10, CCL2, Eotaxin and CCL3 (Figure 31). IFNα significantly altered the proinflammatory cytokine response in comparison to Veh Ctrl and IFNλ2 treatment groups. The cytokine response between Veh Ctrl and IFNλ2 groups were however comparable from day 4 post infection onwards. This suggests that in the context of on-going IAV infection, further administration of IFNα results in decreased viral loads, but also drives immunopathology. In striking contrast, IFNλ does not induce this immunopathology, as it does not modify the proinflammatory cytokine secretion.

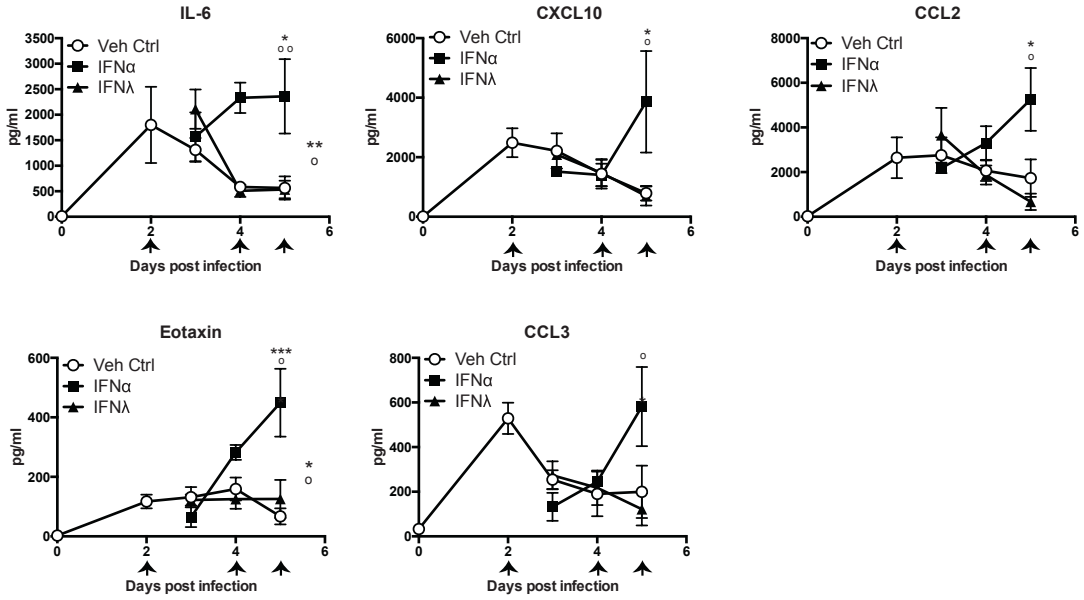


Figure 31 IFNα4 treatment causes increased proinflammatory cytokine secretion during IAV infection

B6.A2g-Mx1 mice were infected with PR8 and treated with IFNα4 (1.45 µg/50µl), IFNλ2 (2.6 µg/50µl) or Veh Ctrl (50ul) as per Figure 30B. IL-6, CXCL10, CCL2, Eotaxin and CCL3 were measured by multiplex cytokine assay from BAL fluid. Arrowheads represent days of treatment. Significance was assessed by 2-way ANOVA with Bonferroni post tests. * indicates IFNα4:Veh Ctrl, and ^{o} indicates IFNα4:IFNλ2. IFNλ2:Veh Ctrl was not significant. Symbols on the right of the graphs indicate statistical significance of the whole curve (2-way ANOVA) while those above indicate significance of individual time points (Bonferroni post test). * P<0.05, ** P<0.01, ***P<0.001. Graphs show mean ± SEM and are representative of two independent experiments, n=3. *Samples collected and processed in collaboration with Dr Sophia Davidson.*

IFNα, IFNβ and IFNλ levels were also measured throughout infection (Figure 32A). The peak of endogenous IFNs is at 2 days post infection and is comparable between the three groups. After administration of IFNα or IFNλ on day 3 post infection, their levels subsequently drop on day 4 post infection. IFNα protein levels on day 5 post infection are significantly increased, whereas they are not in the Veh Ctrl or IFNλ groups. Similarly, IFNλ levels are increased at 5 days post infection, but these are unchanged in the Veh Ctrl and IFNα groups. This may be due to the IFN positive feedback loop or, more likely, to the detection of exogenous IFNs administered on day 5 post infection. To understand if the IFN positive feedback loop is in fact playing a role, IFN transcript levels over time post treatment were assessed (Figure 32B). There is no evidence of a positive feedback loop for endogenous IFN production since the transcript levels for all three IFNs tested are essentially unmodified by IFNα or IFNλ treatment. Also, to ensure the IFNs administered are reaching the lung and show that there is no basal level of IFNs within a naïve mouse, BAL was taken from mice 5 minutes after treatment and measured for IFNα, IFNβ and IFNλ (Figure 32C). We see only protein levels of IFNs corresponding to the treatment administered, whereas we see no IFNβ, as expected. These results indicate that it is the exogenous IFN administered that is driving the observed divergence in disease and proinflammatory cytokine induction.

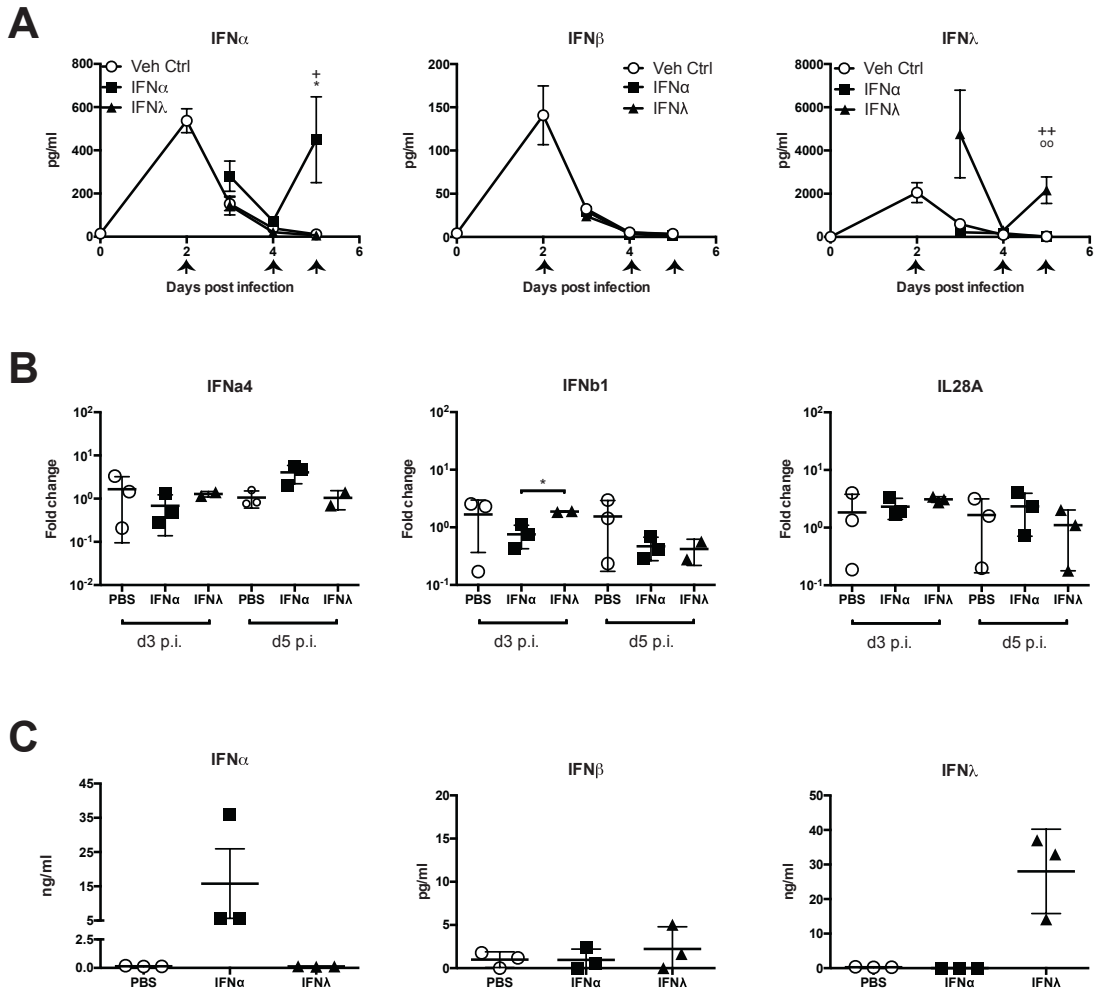


Figure 32 The exogenous administered IFNα4 rather than endogenous IFNα4 is driving the exacerbated disease

B6.A2G-Mx1 mice were infected with PR8 and treated with IFNα4 (1.45µg/50µl), IFNλ2 (2.6µg/50µl) or Veh Ctrl (50ul) as per Figure 30B. IFNα, IFNβ and IFNλ were assessed by ELISA (A). Whole lungs were taken at day 3 and 5 post infection and analysed by qPCR for IFNa4, IFNb1 and IL28A (B). B6.A2G-Mx1 mice were treated with IFNα4 (1.45µg/50µl), IFNλ2 (2.6µg/50µl) or Veh Ctrl (50ul) and BAL fluid taken 5 minutes later and assessed by ELISA for IFNα, IFNβ and IFNλ (C). Arrowheads represent days of treatment. Significance was assessed by 2-way ANOVA with Bonferroni post tests. * P<0.05, and ** P<0.01. Graphs show mean Graphs show mean ± SEM and are representative of two independent experiments, n=3. *Data for A was collected and processed in collaboration with Dr Sophia Davidson*

To determine why the different types of IFN resulted in a divergent ability to protect the mice from IAV, the response of different innate immune cells were assessed. *In vitro* stimulation of whole splenocyte cultures with IFNα, but not with IFNλ, resulted in increased ISG expression: Rsad2 and Oasl2 (Figure 33A), and proinflammatory cytokine secretion: IL-6, CXCL9, CXCL10 and CCL2 (Figure 33B). IFNλ did not induce these ISGs and cytokines due to the lack of IL28Ra on hematopoietic cells (Figure 33C), thus confirming the literature on the distribution of the IFN receptors (Sommereyns *et al.* 2008; Mordstein *et al.* 2010).

As the IL28Ra is present on the non-haematopoietic cells, the response to IFN stimulation on AECs was assessed. AECs are the primary infection targets of IAV and as such are the first responders to infection. Upon stimulation AECs respond comparatively to both types of IFNs by the induction of ISGs, as expected (Figure 34A). However, neither IFNα nor IFNλ induced expression or secretion of proinflammatory cytokines (Figure 34B, C). Together, these results suggest that AEC upregulation of antiviral ISGs upon stimulation with IFNα or IFNλ is sufficient to inhibit IAV replication *in vivo.* As IFNλ does not induce proinflammatory cytokines by either innate immune cells or by the AECs, it appears that they are unnecessary to control infection and therefore are only contributing to immunopathology.

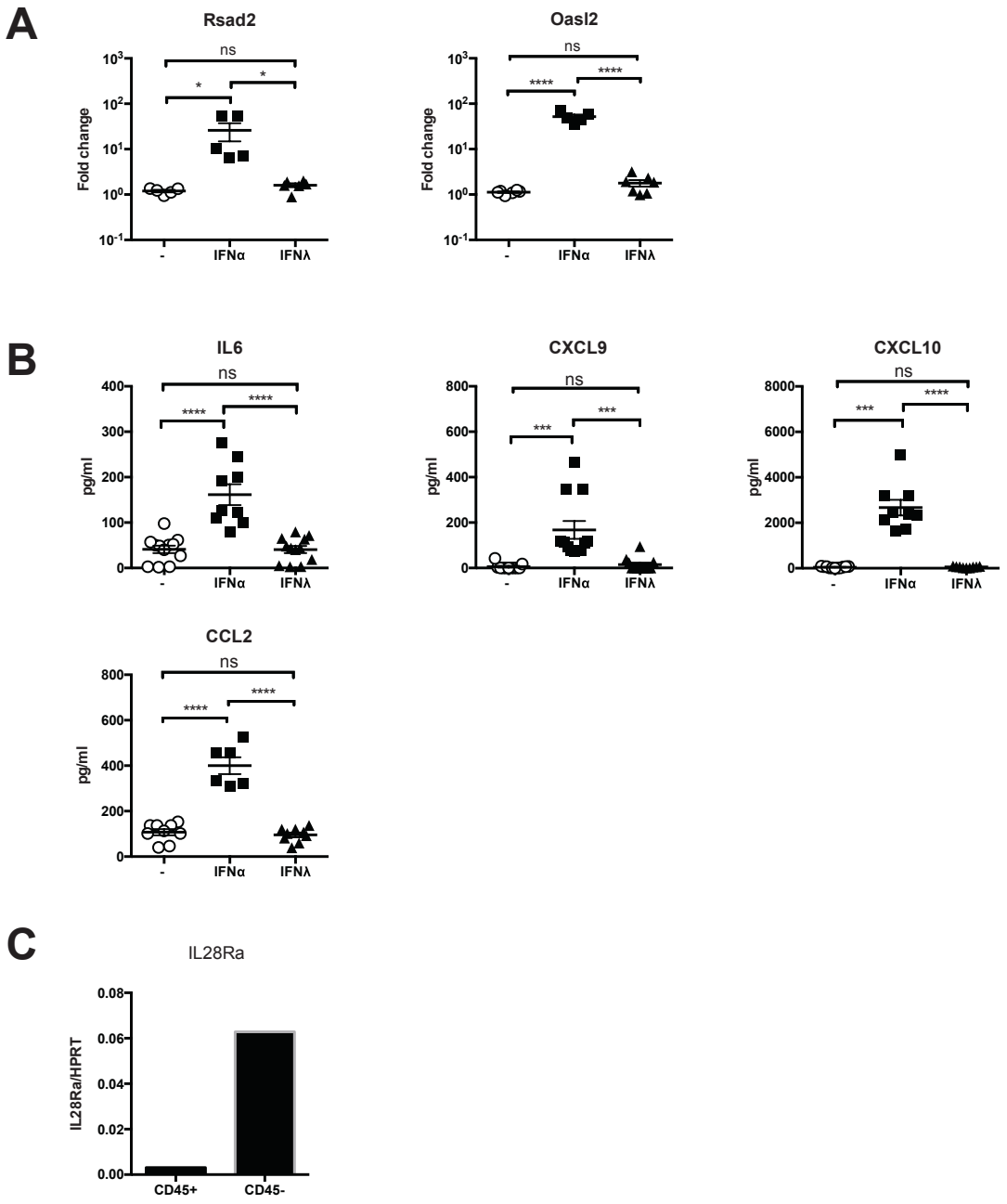


Figure 33 Splenocyte treatment with IFNα, but not with IFNλ, results in ISG and proinflammatory expression

Whole splenocytes isolated from C57BL/6 mice were stimulated with IFNa4, IFNλ or Veh Ctrl for 4 hours. ISG expression was measured by qPCR (A) and proinflammatory cytokine production was analysed from supernatants by multiplex cytokine assay (B). Whole lungs were collected and separated for hematopoietic (CD45+) and non-haematopoietic (CD45-) cells, and IL28Ra expression was measured by qPCR (C) Significance was assessed by unpaired t-tests where * P<0.05, *** P<0.001 and **** P<0.0001. Graphs show mean ± SEM. Data is representative of two independent experiments, n=2-12. *Samples were collected and processed with Dr Sophia Davidson.*

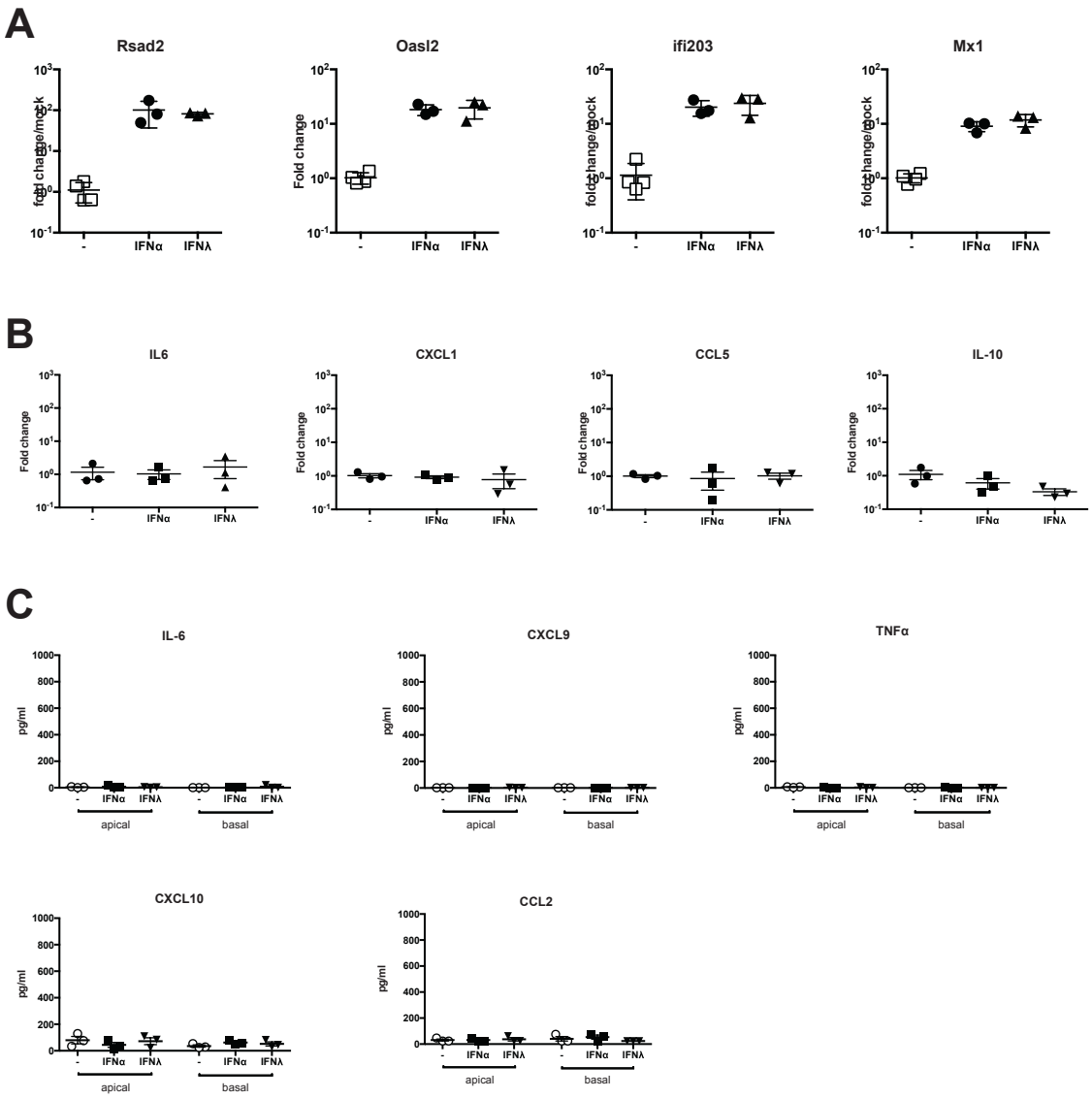


Figure 34 IFNα and IFNλ treatment of AECs induces comparable ISG expression, but no proinflammatory cytokines

C57BL/6 derived AECs were treated with IFNα, IFNλ or Veh Ctrl for 4 hours then assessed for ISGs (A) and proinflammatory cytokines (B) by qPCR. Supernatants were collected and analysed for proinflammatory cytokines by multiplex cytokine assay (C). Graphs show mean ± SEM. Data is representative of two independent experiments, n=2-4.

5.3.3 Human primary epithelial and immune cells show the same divergence in IFNα versus IFNλ responsiveness as mouse cells

To be able to extend our study to human cells, the same determination of equivalent doses of human IFNα and IFNλ had to be carried out. To do this, primary human AECs were generated and treated with a serial dilution of human IFNα or IFNλ, and antiviral ISGs were assessed (Figure 35A). The human AECs responded to both IFNs in a dose-dependent manner. Dose-response curves were then used to determine EC50 values, which allowed for calculation of a conversion ratio of 17.5, for equipotent treatment with IFNα4 and IFNλ2 on human cells, as per Figure 29 (Figure 35B).

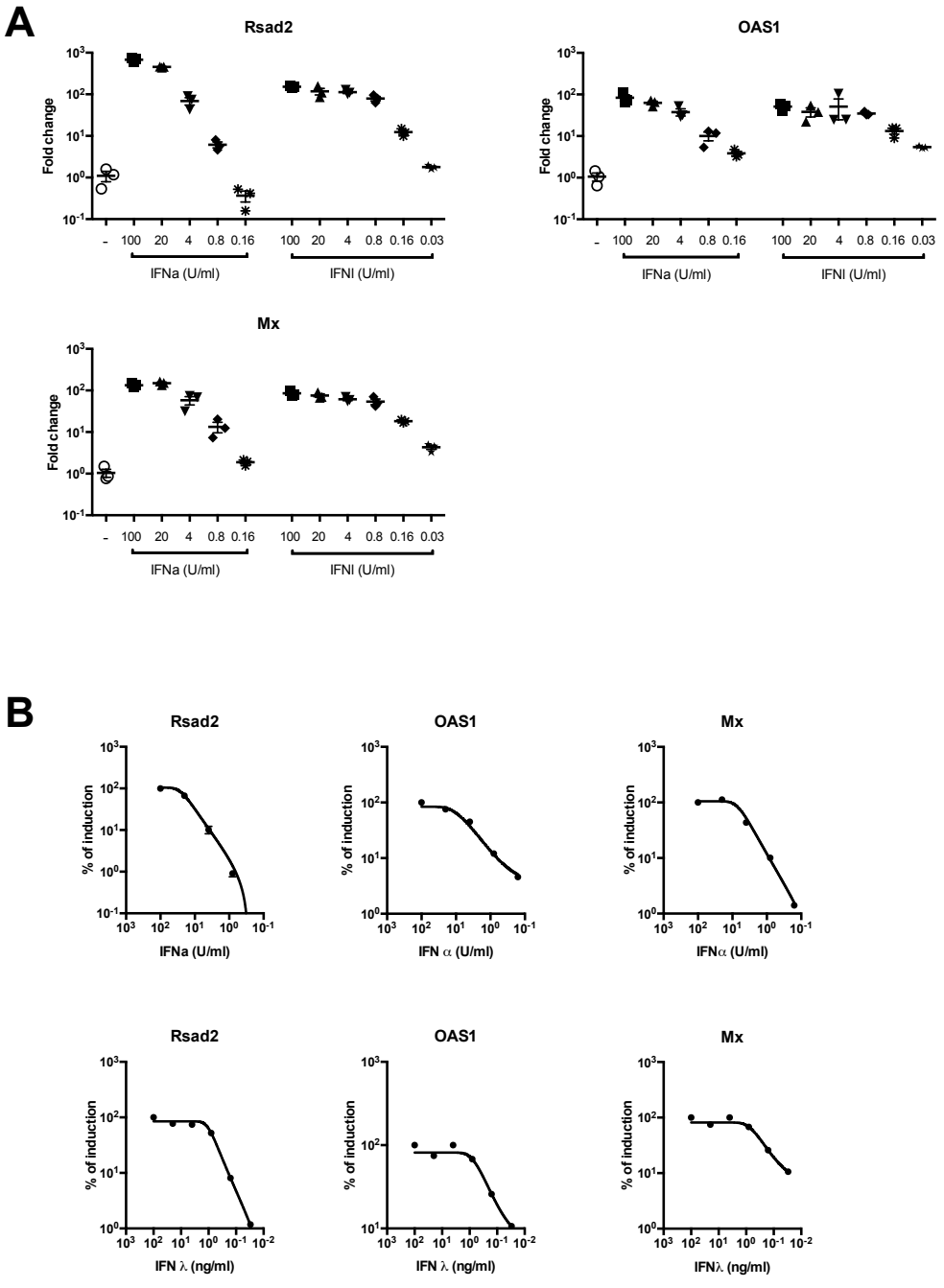


Figure 35 Determination of equivalent doses of IFNα and IFNλ on primary human AECs

Human AECs were stimulated for 4hrs with IFNα4, IFNλ2 or Veh Ctrl, and induction of ISGs were assessed by qPCR. Prism 6 software was used to perform a curve fit (Sigmoidal, 4PL), to generate a dose response curve and to determine a half-maximal effective concentration (EC_{50}) for each gene assessed for each treatment. A conversion ratio was then generated using the formula from Figure 29 for each ISG and a final conversion ratio was taken from the average of all ISGs assessed. Values represent means ± SEM. Data is representative of 2 independent experiments, n=3.

Human peripheral blood mononuclear cells (PBMCs) were collected from healthy donors and assessed for ISG and proinflammatory cytokine induction in response to IFNα and IFNλ after 4 and 24 hours (Figure 36). IFNα induced expression of ISGs (Figure 36A) and proinflammatory expression and secretion (Figure 36B, C) at both time points. Messenger levels of TNFα were not induced by IFNα treatment, however an increase in protein levels was observed, indicating that TNFα expression may have peaked before the first time point tested. IFNλ on the other hand did not induce any of the ISGs or proinflammatory cytokines tested, in accordance to what we saw when mouse cells were used.

To further corroborate that IFNλ treatment during an infection does not induce immunopathology, human PBMCs were infected and treated with IFNα or IFNλ for 24 or 48 hours. PR8 infection of PBMCs induces both ISGs and proinflammatory cytokines at both time points (Figure 37). Further treatment with IFNα or IFNλ along with IAV infection does not enhance ISG expression at 24 or 48 hours (Figure 37A). Similarly IL-6 expression is not enhanced following IFNα or IFNλ treatment (Figure 37B). Cytokine secretion is also not enhanced following IFNα or IFNλ treatment (Figure 37C). Here we are seeing no IFNα driven immunopathology, however these time points differ from the *in vivo* infection and treatment model, as IFNs are given earlier, and ISGs and proinflammatory cytokines are assessed earlier. An *in vitro* model should be set up to completely mimic the *in vivo* model, however the viral-induced apoptosis of the PBMCs is of concern and may thus not fully represent the *in vivo* scenario. Collectively, these results further confirm that IFNλ treatment is unlikely to drive the immunopathology observed with IFNα, making it a more attractive treatment option for humans.

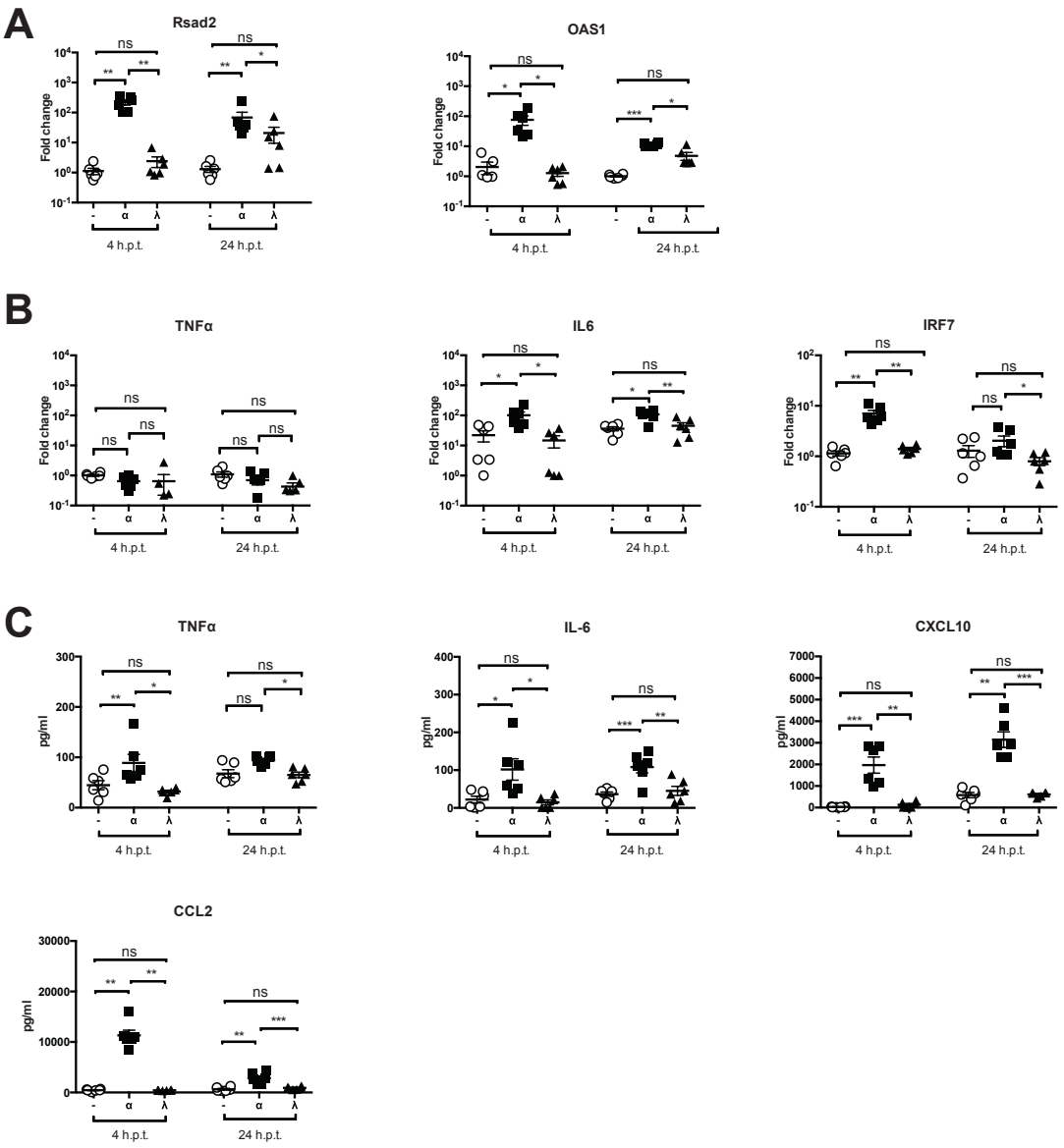


Figure 36 IFNα4 treatment induces ISGs and proinflammatory cytokines in human immune cells, whereas IFNλ does not

ISG (A) and proinflammatory cytokine induction (B) in human PBMCs was assessed at 4 and 24hrs post IFNα (21 U/ml) or IFNλ (1.2 ng/ml) stimulation. PBMC proinflammatory cytokine secretion was measured by multiplex cytokine of supernatants (C). Significance was assessed by 2 way ANOVA where * P<0.05, ** P<0.01, and *** P<0.001. Data shown is representative of 6 independent donors, n=6.

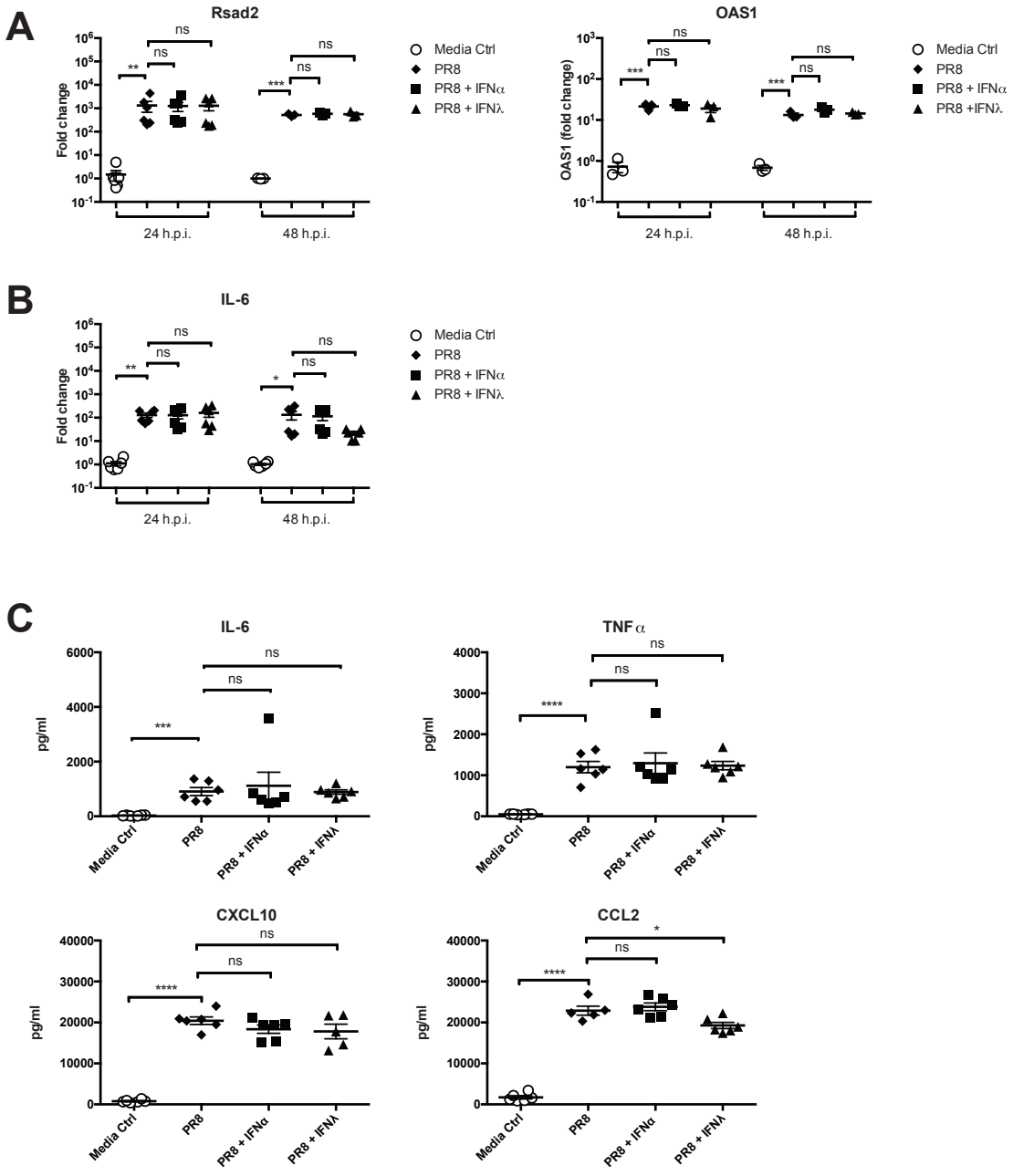


Figure 37 IFN addition during IAV infection on human immune cells does not enhance ISG or proinflammatory cytokine responses

ISG (A) and IL-6 cytokine induction (B) in human PBMCs was assessed at 24 and 48 hours post PR8 infection and treatment with IFNα (21 U/ml) or IFNλ (1.2 ng/ml) stimulation. PBMC proinflammatory cytokine secretion was measured by multiplex cytokine assay of supernatants(C). Significance was assessed by unpaired t test where * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001. Data shown is pooled from 6 independent donors, n=3-6.

5.4 Conclusions and Discussion

An ideal treatment option for IAV should stimulate the induction of antiviral genes in AECs to control the spread of the virus, without driving immunopathology. Both IFNα and IFNλ, if given prior to infection, can control the spread of IAV, however only IFNλ is protective if given during an on-going infection. IFNα treatment resulted in increased proinflammatory cytokine production, recruitment of innate inflammatory cells and apoptotic AECs (data shown here and Davidson *et al.* 2016), thus promoting IAV-induced morbidity and mortality. Treatment of human AECs and PBMCs showed the same dichotomy, with IFNα resulting in increased cytokine production by hPBMCs. Overall, this highlights IFNλ as a stronger candidate for therapeutic application against IAV in humans.

The IFN induced production of ISGs prior to IAV infection blocks the virus from establishing an infection, as seen by the reduced viral loads within the lung compared to the Veh Ctrl group (Davidson *et al.* 2016). Previous studies also showed that pre-treating mice with type I IFNs protected against a variety of IAV strains (Beilharz *et al.* 2007; Tumpey *et al.* 2007), although within the Beilharz *et al.* study, mice displayed increased morbidity if they were given higher doses of IFNα (Beilharz *et al.* 2007). Pre-treating an entire population before a serious IAV outbreak is not realistic. Mice were thus treated from the onset of IAV-symptoms, such as weight loss, at day 2 post infection onwards. Again we see reduced viral loads after treatment from both types of IFN compared to the Veh Ctrl group (Davidson *et al.* 2016). IFNα, IFNβ and IFNλ concentrations within the lung following infection are low. This is likely due to either the B6.A2G-Mx1 mice being on the B6 background which have already been shown to be low IFN responders to IAV (Davidson *et al.* 2014) or to IAV induced blockade of IFNs. NS1 protein of IAV

is known to inhibit IFN induction by interfering with upstream pathways such as blocking RIG-I ubiquitination or IRF-3 activation (Gack *et al.* 2007; Hale *et al.* 2008). The addition of exogenous IFN may therefore control IAV replication through the increased expression of ISGs or by bypassing the blockade induced by IAV NS1 protein (Ehrhardt *et al.* 2010).

Virus control following treatment did not always correlate with disease outcome, as IFNα treated mice showed increased morbidity and mortality despite reduced viral load. Within humans, severe IAV disease is characterised by a 'cytokine storm' and pulmonary tissue destruction, which can be host- or immune-mediated (Peiris *et al.* 2004; de Jong *et al.* 2006; Louie *et al.* 2009). Exogenous IFNα treatment provoked increased secretion of proinflammatory cytokines such as IL-6 and CCL2 when compared to the Veh Ctrl or IFNλ treatments. Increased recruitment of immune cells such as pDCs and inflammatory monocytes, and AEC apoptosis was also observed following IFNα treatment (data shown in Davidson *et al.* 2016). pDCs and IMcs can secrete proinflammatory cytokines and chemokines such as CCL2 and IFNαβ following infection. Therefore, enhanced recruitment could propagate the proinflammatory cytokine secretion, resulting in amplified lung inflammation leading to AEC damage and ultimately mortality of the host. Furthermore, *in vitro* treatment of whole splenocytes with IFNα, but not with IFNλ, drives proinflammatory cytokine secretion.

Type I IFN induced inflammation and epithelial cell death is designed to be protective for the host. Recruitment of immune cells and subsequent cell death of infected cells should contribute to viral clearance. However, if this system becomes over activated, such as from the addition of more IFNα, then increased recruitment, cytokine production and lung damage occurs. To identify the overlaps and

differences in the lung transcriptional response to both IFNα and IFNλ, we performed microarray analysis on whole lungs treated with Veh ctrl, IFNα or IFNλ. Samples were normalized to the average of the Veh ctrl and filtered for a fold change of 1.5 which yielded 553 genes differently expressed between the two treatments, of which 429 were upregulated. Upon K-means clustering of the genes upregulated we obtained six gene clusters induced by IFNα alone and the remaining genes were induced by both IFNα and IFNλ. IPA analysis of the IFNα specific genes revealed the we have found that the "Role of hypercytokinemia/hyperchemokinemia in the pathogenesis of influenza" as one of the top pathways induced, while the pathways common to both treatments were strongly related to IFN signalling pathways (Davidson *et al.* 2016). These genes are, unsurprisingly, primarily proinflammatory cytokines, thus concurrent with human studies which found mortality associated with hypercytokinemia (Hayden *et al.* 1998; Peiris *et al.* 2004; de Jong *et al.* 2006; Yang *et al.* 2012). IFNα and IFNλ treatment induce a highly similar set of genes in AECs (Crotta *et al.* 2013), in fact we see similar ISG expression but no proinflammatory cytokine secretion by either treatment of AECs *in vitro*. Restricted IFNλR allows IFNλ treatment to target the cells most at risk to IAV infection, without exacerbating the production of proinflammatory cytokines. Therefore, it may also be advantageous within this system to block type I IFN signalling late in infection.

Collectively, these results suggest that it is the IFNα driven proinflammatory cytokine production by immune cells that is driving AEC damage during an active IAV infection resulting in host mortality. In contrast, IFNλ treatment does not drive a cytokine storm, and the ISG induction in AECs is sufficient to control IAV replication and protect the host from IAV-induced disease. To completely conclude that it is in

fact the exaggerated proinflammatory cytokine response that leads to mortality of the host, a blocking antibody such as anti-mouse Ly-6C/G (Gr-1) or anti-PDCA1, could be used along with IFNα treatment. Since this should block immune cells recruitment, IFNα treatment should lose its immunostimulatory effect and lead to the same outcome as with IFNλ treatment. Generating bone marrow chimeras of B6.A2G-Mx1 mice reconstituted with an IFNαβR sufficient or deficient hematopoietic system would be an alternative strategy to assess whether IFNα4 treatment drives the excessive immunopathology specifically in immune cells. Chimeras reconstituted with IFNαβR-/- hematopoietic system should be protected whereas mice receiving a Wt immune system would have increased morbidity and mortality. A caveat to this is that radio-resistant myeloid cells may still be present within the lung, which could potentially contribute to epithelial cell apoptosis. However a study by Hogner *et al.* showed that Wt C57BL/6 mice reconstituted with TRAIL-/- or IFNαβR-/- immune system were protected from IFNαβ/TRAIL induced cell death (Högner *et al.* 2013). Another caveat is the possibility that pulmonary endothelial cells may be a source of IFNαβ driven proinflammatory cytokines (Sommereyns *et al.* 2008; Teijaro *et al.* 2011). If this is possible then IFNα4 treatment could still induce inflammation by stimulating these endothelial cells to induce immunopathology.

Pre-treating with IFNα or IFNλ does not confer protection against all viral infections. Pre-treatment with IFNα was more effective against encephalomyocarditis virus (ECMV) and LCMV, whereas IFNλ was more effective against herpes simplex virus type 2 (HSV-2) (Ank *et al.* 2006). This variation in protection is likely due to the virus tissue tropism as IFNλR is restricted primarily to mucosal surfaces such as the lung epithelial layer (Sheppard *et al.* 2002; Mordstein

et al. 2008). IFNλ is thus effective against viruses that preferentially replicate within the epithelium, whereas it is less effective against viruses that replicate in other cell types. Thus in our system of IAV infection, IFNλ is the preferential treatment as IAV replication is restricted to the AECs. Although IFNα stimulation over-activates the immune response in this setting, it may be protective in other virus infections that preferentially replicate within immune cells. Furthermore, we treated mice that had a complete, healthy immune system, whereas many fatalities from influenza infection occur in immunocompromised individuals who may benefit from immune cell stimulation, in which IFNα could be the appropriate treatment option. A number of studies have shown that some strains of IAV can replicate within immune cells such as AMs and DCs *in vitro*, however this is usually non productive (Rodgers and Mims 1982; Perrone *et al.* 2008). Human monocyte derived macrophages or DCs have also been reported to be infected by highly pathogenic H5N1 or 1918 IAV strains (Perrone *et al.* 2008; van Riel *et al.* 2011).

A number of studies have shown that some immune cells do indeed respond to IFNλ stimulation (Jordan *et al.* 2007), such as DCs, NK cells and macrophages (Ank *et al.* 2008; Liu *et al.* 2011). IFNλ was also found to reduce neutrophil numbers and IL-1β production in Th17-driven diseases (Blazek *et al.* 2015). Neutrophils express IFNAR, as all immune cells do, however they are unique in their expression of the IFNλR (ImmGene database). The IFNλ effects Blazek and colleagues describe would equally be mediated by IFNα (Davidson *et al.* 2015) and are most likely not important in our system since we found no significant changes in neutrophil numbers or IL-1β levels by either IFN treatment. To fully assess the role of IFNλ and its potential protective abilities, different strains

of IAV, particularly those capable of replicating within the immune cells, such as highly pathogenic H5N1, should also be studied.

The pathogenic potential of type I IFNs has been previously reported in mice that lacked a functional Mx1 protein (Davidson *et al.* 2014). As the human homologue of Mx1, MxA, has been shown to be a potent restrictor of IAV both *in vivo* and *in vitro* (Pavlovic *et al.* 1995), the mice used in this study are a more appropriate model for comparisons to human IAV infections. IFNα driven immunopathology was nonetheless demonstrated to outweigh the protective effect of the IFN-induced anti-IAV Mx1 protein (Horisberger 1995). However, mouse models, unlike humans, are naïve to a multitude of pathogens and it is consequently unknown whether this study will translate into a clinical application. Natural mutations within humans that are linked to IFN production, for example IRF7 and IFITM3 (Everitt *et al.* 2012; Ciancanelli *et al.* 2015), could impair the effectiveness of IFNλ treatment, whilst other mutations which enhance STAT1 (Yamazaki *et al.* 2014) activity could over activate the IFN response, thus rendering IFNλ useless. IFNλ has already been utilised as a treatment against hepatitis C patients, in which it was found that IFNλ was just as effective as IFNα, but with reduced hematologic toxicities such as neutropenia or thrombocytopenia and fewer influenza-like symptoms such as chills, pyrexia or pain (Ramos 2010; Donnelly *et al.* 2011; Muir *et al.* 2014). Since many influenza-like symptoms are caused by proinflammatory cytokine secretion and inflammation (Eccles 2005), this further validates the lower direct immunomodulatory effect of IFNλ compared to IFNα also in humans.

As IFNλ has been employed as a treatment in other diseases but not with IAV, we therefore replicated our data on human AECs and human PBMCs. Both

IFNα and IFNλ induced ISG expression in AECs as expected, but only IFNα induced cytokine secretion from PBMCs, as was observed with the mouse cells. IFN treatment of IAV infected PBMCs did not result in enhanced ISG or cytokine secretion regardless on the type of IFN given, but a longer time point should be assessed. Interestingly, there appears to be a slight modulation of CCL2 following IFNλ treatment of IAV infected PBMCs. This could hint at the possible responsiveness of some immune cells to IFNλ, in agreement with previous studies mentioned (Ank *et al.* 2008; Liu *et al.* 2011; Blazek *et al.* 2015). Although immune cells may be responsive to IFNλ under some circumstances, it is encouraging that IFNλ treatment does not contribute to a 'cytokine storm' within these cells. Overall, our human data suggests that IFNλ does not induce cytokine production within human immune cells, unlike IFNα.

In conclusion, from the results presented in this chapter, it is clear that IFNλ is a preferential IAV treatment option, and that IFNα treatment should only be used with caution. IFNα acts on all cells types to induce an excessive immune response resulting in a 'cytokine storm' and host pathology. IFNλ, owing to its receptor distribution, only acts upon the first host cell target of infection to induce a sufficient antiviral response within these cells to control infection, without inducing immune-mediated pathology.

Chapter 6. Antiproliferative effect of IFNs on regenerating epithelia

6.1 Background

Since its discovery in 1957, type I IFNs were classically described as antiviral cytokines (Isaacs and Lindenmann 1957). However, since it was first described in 1962, the antiproliferative effect of type I IFNs has been extensively studied (Paucker *et al.* 1963). Paucker and colleagues showed a temporary decline in the growth of cells following a 24 hour exposure of L-cells to either IFN or UV-irradiated Newcastle Disease Virus (Paucker *et al.* 1963). Furthermore, IFN addition was found to inhibit the growth of the oncogenic Friend virus, Rauscher virus and Mouse Sarcoma virus (MSV) along with inhibiting the cellular transformation induced by these viruses *in vitro* (Oxman *et al.* 1967; Fitzgerald 1969).

These mouse studies prompted considerable work on the effects of interferon on human malignancies. An example of this translational research is the delay in mammary tumour development found when female mice were treated with IFN (Came and Moore 1972). Balkwill and colleagues then advanced from there to discover that two out of three human breast cancers were susceptible to IFN treatment following xenograft implantation in athymic nude mice (Balkwill *et al.* 1980). Prompted by these encouraging results, clinical trials were conducted on the effect of type I IFN treatment on malignant melanoma, acute granulocytic leukemia, multiple myeloma and chronic lymphatic leukemia, all of which displayed some tumour regression or delay in tumour growth following IFN treatment. The first licensed interferon for anti-tumour applications was IFNα2 for the treatment of Hairy

Cell Leukemia (HCL) in 1986 (Golomb *et al.* 1986). Subsequently, IFNα2a (Roferon-A, Hoffman-La Roche, Nutley, NJ) and IFNα2b (Intron-A®, Schering-Plough, Kenilworth, NJ) have been licensed for the treatment of: Chronic Myelogenous Leukemia (Talpaz *et al.* 1987; Kikawa *et al.* 1993; Guilhot *et al.* 2004), Follicular Lymphoma (Aurora and Winter 2006), Malignant Melanoma (Sabel and Sondak 2003), and AIDS-Related Kaposi's Sarcoma (Krown *et al.* 2002).

The mechanism(s) by which these IFNs induce their antiproliferative effects have yet to be fully elucidated and are a matter of continuous study. Type I IFNs are known to affect different phases of the mitotic cell cycle, specifically during the S phase in solid tumours and G1 arrest in Daudi Burkitt's lymphoma cells (Roos *et al.* 1984; Garrison *et al.* 1996; Qin *et al.* 2001). The downregulation of the transcription factor c-myc by type I IFN was also found to induce cell-cycle arrest (Einat *et al.* 1985). Furthermore, IFN treatment was shown to upregulate the tumour suppressor gene p53 which is known to initiate cycle-arrest or apoptotic pathways (Oren 1994; Takaoka *et al.* 2003). Other pro-apoptotic effect of IFNs are the upregulation of apoptotic proteins such as DR5 and TRAIL resulting in apoptosis of DR5 expressing cells (Herold *et al.* 2008; Davidson *et al.* 2014).

Not only can IFNs be directly antiproliferative or pro-apoptotic but they can also result in modulation of growth factor expression. Epidermal growth factors (EGF) and fibroblast growth factors (FGF) are required for the proliferation of cells, which is particularly important for repair. The production of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) was found to be reduced following IFN treatment (Singh *et al.* 1995; Marshall and Swain 2011). Growth factors of the EGF family exert their action through the Epidermal Growth Factor Receptor (EGFR) and IFNα treatment of renal carcinoma cells was found to

downregulate EGFR to induce its antiproliferative effect (Eisenkraft *et al.* 1991; Scambia *et al.* 1991). In contrast, other studies have found that EGFR expression was enhanced following IFNα and IFNβ treatment in some tumour cells, which antagonized the antiproliferative effect of type I IFNs (Martyré *et al.* 1990; Caraglia *et al.* 1995; Yang *et al.* 2004). These conflicting reports may be due to the different cancer cells studied, different IFNα subtype and concentrations used, and also different timings of treatments. Nonetheless, Zoon *et al*. also found that IFNα treatment reduced the ability of EGF to bind to EGFR leading to the antiproliferative effect of IFNα (Zoon *et al.* 1986). The effect of type I IFN on innate immune cells which have cytotoxic effects has already been discussed in Chapter 3. Whether it is anti-proliferative, pro-apoptotic, immunmodulatory effects, or a combination of all three that underlie the anti-tumour action of IFN is not precisely known, and the effect of type I IFN to elicit these mechanisms may change according to the type of tumour and the varying responsiveness of different cell types to type I IFN.

The recently discovered type III IFNs (Kotenko *et al.* 2002), IFNλ1 (IL-29), IFNλ2 (IL28A), IFNλ3 (IL28B) and IFNλ4 have previously been discussed to induce the same signalling pathways as the type I IFNs, and their antiproliferative and anti-tumour abilities have recently gained attention (Dumoutier *et al.* 2004; Brand 2005; Maher *et al.* 2014). The mechanisms of the antiproliferative effects of IFNλ are still unclear, and have not been as extensively researched as type I IFN effects. Not only was IFNλ found to be antiviral against murine cytomegalo virus (MCMV), but was also found to decrease the proliferation of intestinal epithelial cells *in vitro* without an effect on apoptosis (Brand 2005). Maher and colleagues found that IFNλ treatment resulted in stronger and prolonged downstream signalling compared to IFNα treatment, and in contrast to the previous study, this prolonged signalling

resulted in apoptosis rather than growth inhibition of human keratinocyte HaCaT cell line (Maher *et al.* 2014). This divergence in results could be due to the different cell types studied: intestinal epithelial cells and keratinocytes, in which IFNλ may be mediating different effects. Dumoutier and colleagues did not quantify apoptosis of cells but suggest that STAT2 activation is required for the antiproliferative effects of IFNλ (Dumoutier *et al.* 2004). IFNλ as an anti-tumour treatment has as of yet not been employed in clinical trials.

A hallmark of influenza is productive infection by IAV and the subsequent destruction of the airway epithelium (Mauad *et al.* 2010). The AECs respond to infection by the induction of type I and III IFNs, a family of pleiotropic cytokines which subsequently induce antiviral ISGs to control the spread of the virus (Randall and Goodbourn 2008; Vareille *et al.* 2011). Once the virus is eliminated, the airway epithelium must undergo extensive mitosis in order to repair and return back to homeostasis. Currently, the antiproliferative effects of IFNs have largely only been studied as anti-tumour therapies. However, the known antiproliferative effect of IFNs poses the question of whether it can affect the repair of lung AECs, which at present has not been assessed. As repair of the lung epithelium after infection is essential, the prolonged presence of IFNs with antiproliferative and pro-apoptotic effects may therefore prove to be detrimental to the host. The genetic contribution to this repair process has already been discussed in Chapter 4 where 129S8 mice were shown to have reduced ability to repair following injury. Since these mice are known to be high type I IFN producers (Davidson *et al.* 2014), how this repair process is affected in the presence of high type I IFN levels is of interest. Due to the tissue tropism of IFNλR, the role of IFNλ on regenerating epithelia becomes particularly intriguing since IFNλ acts primarily on AECs, and shown in Chapter 5 it

is the most suitable antiviral therapeutic in IAV infection. Could the additional exogenous IFNλ treatment potentially disrupt repair of AECs? As such the effect of type I and type III IFNs on regenerating AECs was assessed.

6.2 Hypothesis and Aims

Given the antiproliferative role of type I and III IFNs, their presence may interfere with the repair process following IAV-induced lung damage. Any disruption to the ability of the lung to repair could lead to impaired lung function, or could potentially leave the host vulnerable to other pathogens. Both of the scenarios could lead to increased morbidity and mortality of the host. Plating of AECs can be considered a model of epithelial repair, a process to reach confluence again and differentiate into the constituent cell types. To determine the antiproliferative effect on the repair process of epithelia, type I and III IFNs will be added to C57BL/6 AEC cultures from start of culture.

I hypothesize that the addition of type I and III IFNs to growing epithelia will inhibit the growth of the AECs through a reduction in EGF signalling.

6.3 Results

6.3.1 Antiproliferative and remodelling effects of IFNs on regenerating AECs

Before the antiproliferative effects of type I and III IFNs can be tested and compared on regenerating epithelia, equivalent amounts of IFNα, IFNβ and IFNλ must be determined. The mouse IFNλ used in Chapter 5 was purified and generously gifted by Dr R. Hartmann, whereas the IFNλ used for this Chapter (and also in Chapter 3) was purchased from R&D systems. Therefore, another serial dilution of the three IFNs to assess equivalent induction of ISGs was performed (Figure 38). The serial dilutions were not performed concurrently. AECs were treated with a serial dilution of either IFNα or IFNβ (Figure 38A), or treated with a serial dilution of either IFNβ or IFNλ (Figure 38B). All AECs respond to the three types of IFN by their induction of Rsad2, Oasl2 and ifi203. The dose response curves were then used to determine EC50 values, which allowed for generation of a conversion ratio of equipotency as per Chapter 5, Figure 30. The conversion ratio for IFNα:IFNβ:IFNλ is 2:1:1 (U/ml:U/ml:ng/ml).

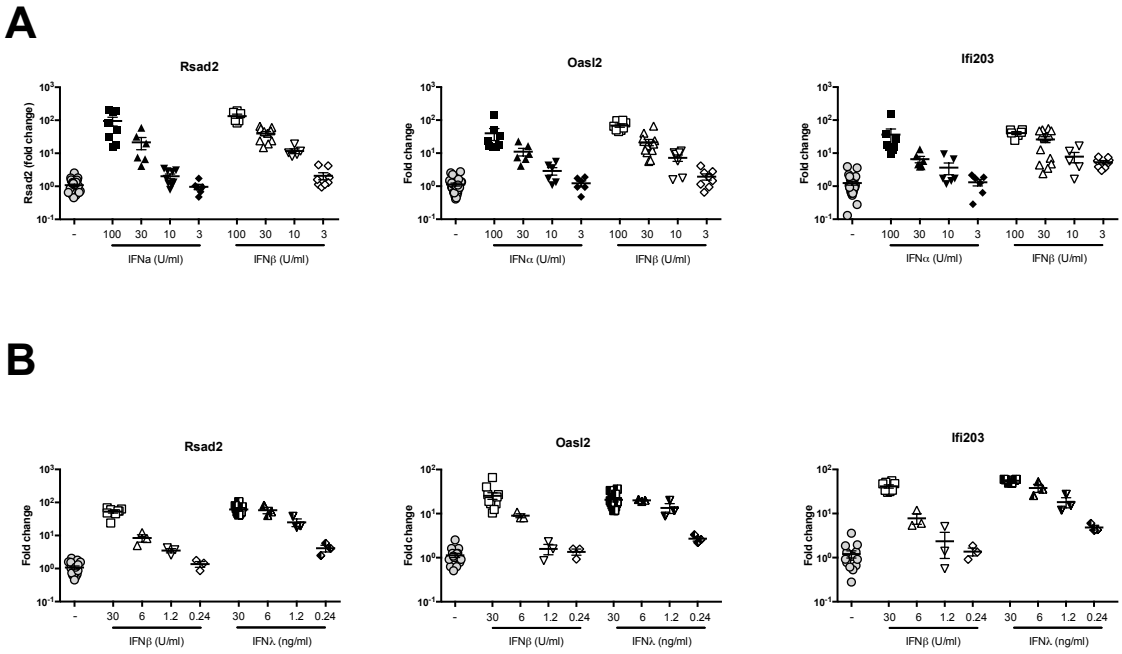


Figure 38 Determination of equivalent doses of type I and III IFNs

C57BL/6 AECs were stimulated for 4hrs with a serial dilution of IFNα4, IFNβ or Media Ctrl (A), and IFNβ, IFNλ or Media Ctrl (B), and induction of ISGs were assessed by qPCR. Prism 6 software was used to perform a curve fit (Sigmoidal, 4PL), and generate a dose response curve and a half-maximal effective concentration (EC_{50}) for each gene assessed for each treatment. A conversion ratio was then generated using the formula from Figure 29 for each ISG and a final conversion ratio was calculated as the geometric mean of all ISGs conversion ratios determined. Graphs show means ± SEM. Data is pooled from 10 independent experiments, n=5-34 (A). Data is representative of 8 independent experiments, n=3-17 (B).

To determine the antiproliferative effect of type I and III IFNs, cells on regenerating airway epithelia, cells were digested out of the trachea and plated in the presence of IFNα, IFNβ, IFNλ or Media Ctrl (Figure 39A). Upon treatment with a serial dilution of IFNβ or IFNλ we find the AECs were unable to reach confluence at any concentration used. However, this effect was not seen when an IFNα concentration equivalent to the highest IFNβ or IFNλ concentration was used, i.e. 60 U/ml IFNα versus 30 U/ml IFNβ and 30 ng/ml IFNλ. (Figure 39B). This effect also correlates with a reduction in cell number on day 14 of culture (Figure 39C). Digesting cells out of the trachea and subsequent plating results in the outgrowth of an initially small subset of basal cells, while differentiated cells most likely undergo apoptosis after plating. These initially infrequent basal cells proliferate allowing the culture to reach confluence. IFNβ and IFNλ addition is preventing this small subset of cells from proliferating. Therefore, to generate a culture where the effect of IFNβ and IFNλ directly on basal cells can be studied and fully quantified, the cultures were allowed to grow as normal without any IFN addition. This allows for the basal cells to proliferate unimpeded, resulting in a large subset of basal cells (Figure 39D). These AECs are digested off the transwells and re-plated at defined numbers in the presence of IFNα, IFNβ and IFNλ (Figure 39E). Here we see a similar outcome following IFNβ and IFNλ addition during growth, although the effect is somewhat diminished, which is most likely due to the increased number of basal cells being plated per well with this approach. A subset of cultures do not reach confluence, which correlates with a reduction in cell number (Figure 39F, G). However, some cultures can reach confluence when the cell number is increased, although the time taken to reach confluence is slower and total cell number is lower than in the Media Ctrl and the IFNα treated cultures (Figure 39F, G).

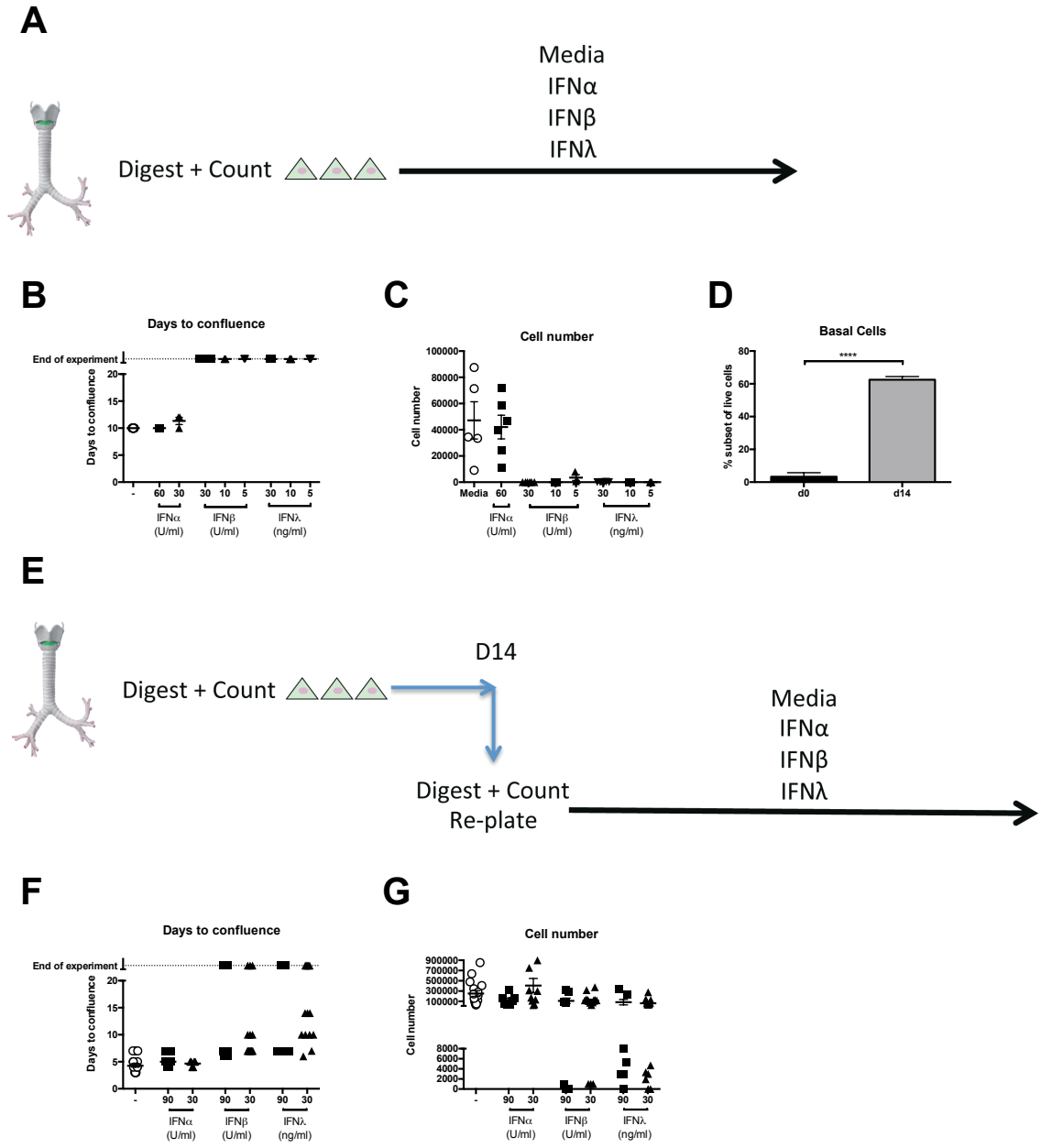


Figure 39 IFNβ and IFNλ prevent the AECs from proliferating and reaching confluence

Tracheas from C57BL6 mice were digested, plated and treated with IFNα, IFNβ or IFNλ (A). Time taken to reach confluence (B) and cell number was quantified on day 14 after plating (C). Percentage of basal cells from cells digested out of trachea before plating (d0) and after the cultures are fully-grown (d14) was quantified by Flow Cytometry (D). AECs were grown as normal for 14 days then digested and re-plated in the presence of IFNα, IFNβ and IFNλ (E). Time taken to reach confluence (F) and cell number was quantified at the end of the experiment (F). Significance was assessed by unpaired t tests where **** P<0.0001. Graphs show means ± SEM. Data is pooled from 2 independent experiments, n=3-11 (B, C), n=4-15 (D). Data is pooled from 8 independent experiments, n=7-24 (F, G).

To further characterize the effect of IFN addition on the different developing cell types in the AECs, the re-plated cultures were analysed by flow cytometry as per Chapter 4, Figure 25. When equivalent amounts of the three IFNs were compared, we see little effect by IFNα on the frequency of basal cells, whereas IFNβ and IFNλ reduce the percentage of basal cells in the cultures (Figure 40A), with IFNλ inducing a statistically significant decrease. All three IFNs reduce the percentage of differentiated cells, with IFNλ again inducing the greatest effect. IFNβ induces an increased percentage of CD49f hi single positive cells, while the IFNλ induced increase is statistically significant (Figure 40A). These CD49fhi single positive cells were observed in Chapter 4 and are as of yet an unknown population. To determine if increased concentrations of IFNs change the phenotype further, a serial dilution was performed. Equipotent doses between all three IFN subtypes were used at the lowest doses: 10 U/ml IFNα, 5 U/ml IFNβ and 5 ng/ml IFNλ, but the highest doses were numerically the same. This is due to this experiment being carried out before the equipotent doses were determined. Increasing the concentration of IFNα does not induce a significant change in AEC subsets; in fact IFNα treated cultures generally appear very similar to the Media Ctrl cultures (Figure 40B). Although the highest dose of IFNα (270 U/ml) is not equipotent to any of the IFNβ and IFNλ doses used here, it is not inducing an effect on the cell subsets in comparison its closest equipotent dose of 90 U/ml of IFNβ and 90 ng/ml of IFNλ. IFNβ and IFNλ however show a dose dependent response with the highest equipotent concentrations having the greatest effect. High IFNβ concentrations cause a dramatic decrease in basal cells and an increase in CD49fhi and undifferentiated cells (Figure 40C). IFNλ also decreases basal cells and differentiated cells, along with an increase in CD49fhi cells (Figure 40D). These

effects start at a much lower concentration than with IFNλ than with IFNβ. This change in cell subsets is much more dramatically observed in the individual flow cytometric analysis plots when equipotent doses of the IFNs are used (Figure 40E). Here we see that in the presence of IFNα the different cell subsets are unaffected and look very similar to the Media Ctrl. IFNβ and IFNλ however show a loss of NGFR/CD49f double positive cells, which we have classified as basal cells (see Chapter 4), and a loss in NGFR/CD49f double negative cells, resulting in a dramatic loss of differentiated cells (NGFR/CD49f double negative, CD133 /CD66a high). There also appears to be a shift of the cells towards the CD49f hi single population. These results suggest that IFNβ and IFNλ addition reduces the proliferation of basal cells and the differentiation of the basal cells available, whereas IFNα does not strongly affect the AECs. IFNλ may have more of an antiproliferative potential as it induces a greater effect at lower concentrations compared to the concentrations of IFNβ.

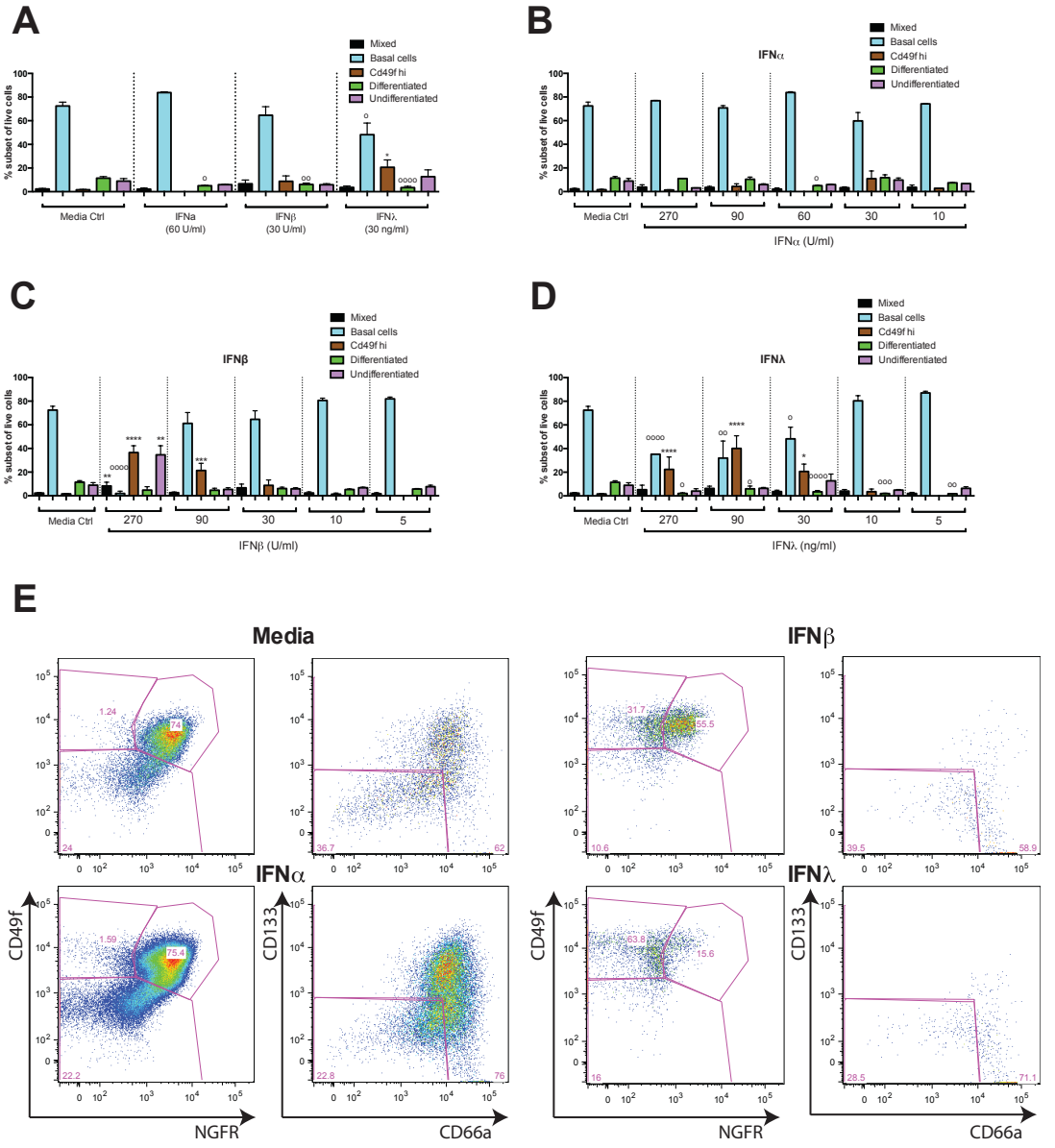


Figure 40 IFNβ and IFNλ treatment causes remodelling of regenerating AEC

Quantification of undifferentiated cells, mixed cells, basal cells, differentiated cells and CD49fhi cells by flow cytometry on AECs following equivalent doses of IFNα, IFNβ or IFNλ from day 0 of re-plating (A). A serial dilution of IFNα was added to re-plated AECs and the different cell subsets were quantified by flow cytometry (B). A serial dilution of IFNβ was added to re-plated AECs and the different cell subsets were quantified by flow cytometry (C). A serial dilution of IFNα was added to re-plated AECs and the different cell subsets were quantified by flow cytometry (D). Flow cytometry plots representative of one sample from AEC treated with equivalent doses of IFNα, IFNβ and IFNλ. The first flow cytometry plot shows how the cells are first subdivided for CD49f and NGFR. The second FACs plot is the CD49f/NGFR double negative population subdivided for CD133 and CD66a (E). Significance was assessed by unpaired t tests. * represents an increase compared

Given the relative reduction of differentiated cells following IFNβ and IFNλ treatment, we wanted to define which of the differentiated cells are affected since our FACS panel cannot distinguish goblet cells and ciliated cells (see Chapter 4). We therefore stained these cultures for β-Tubulin IV, to quantify ciliated cells. Ciliated cells were reduced when the highest equivalent concentrations of IFNα, IFNβ and IFNλ are used. However, when concentrations are reduced, IFNα and IFNβ have less of an effect, whereas IFNλ continues to reduce ciliated cells (Figure 41A). When this is quantified we see only a significant decrease in ciliated cells when the highest concentration of IFNα or IFNβ are used, while, even if the doses are equivalently titrated down further, IFNλ is still affecting the ciliated cells (Figure 41B). This indicates that type I and III IFNs can reduce the differentiation of AECs into ciliated cells, with IFNλ having the greatest effect.

to the equivalent cell subset from Media Ctrl. ^{O} represent a decreased compared to the equivalent cell subset from Media Ctrl. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Graphs show means ± SEM. Data is pooled from 6 independent experiments, n=2-33 (A-D).

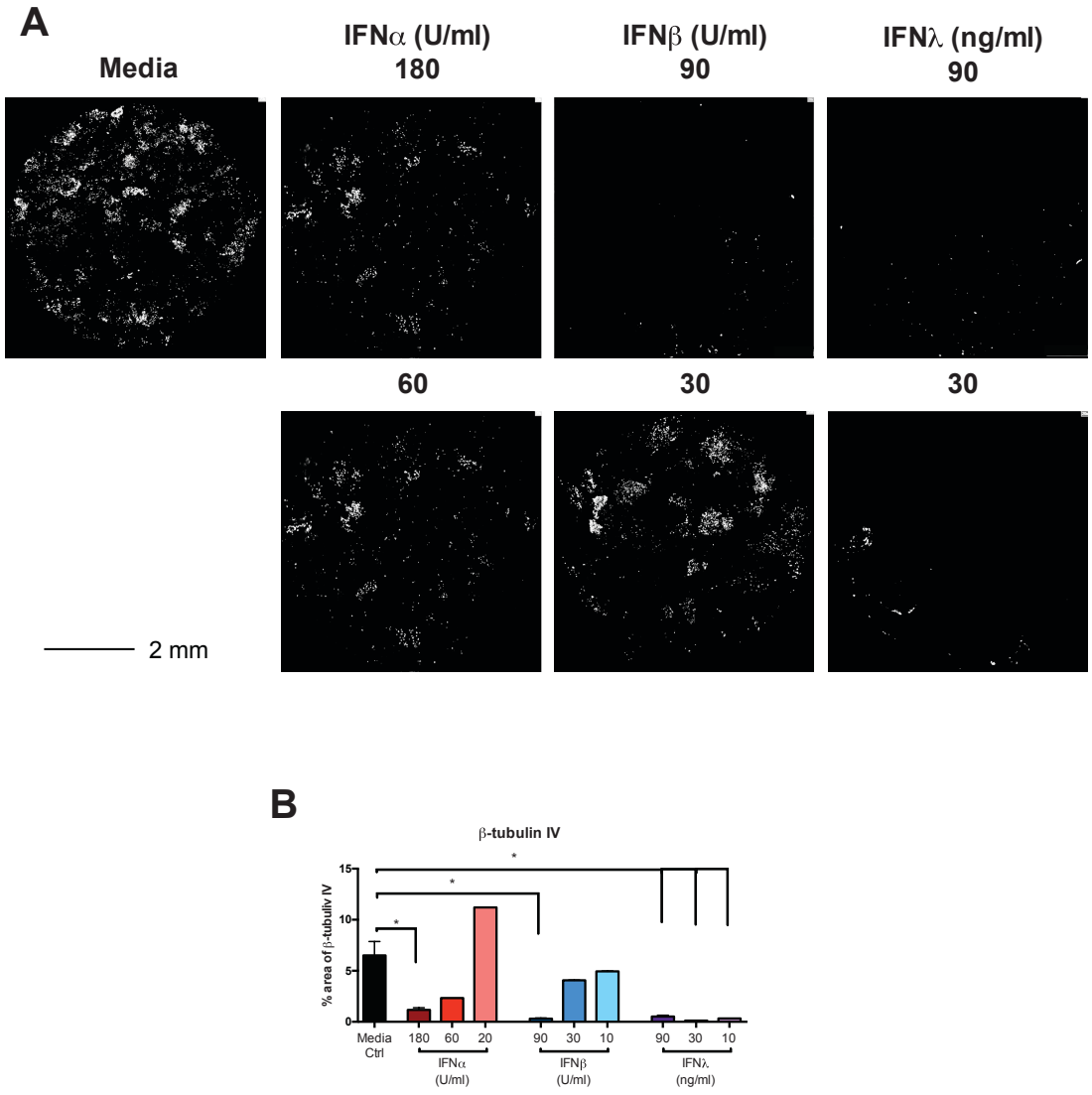


Figure 41 IFN addition during growth of AECs reduces the ability to differentiate into ciliated cells

β-tubulin IV staining (white) of re-plated cultures grown in the presence of equivalent doses of IFNα, IFNβ, IFNλ or Media Ctrl (A). Quantification of percentage area of β-tubulin IV (B). Graph show means ± SEM. Significance was assessed by unpaired t tests where * P<0.05. Data representative of 3 independent experiments, n=1 (A). Data is pooled from 3 independent experiments, n=2-3 (B).

6.3.2 IFN addition to regenerating AECs can induce cell death and apoptosis pathways

It is interesting to see a dose-dependent effect between the three IFNs used, with IFNλ having the greatest and IFNα having the least effect on multiciliation and IFNβ and IFNλ, but not IFNα, greatly reducing cell number increase. To discern whether the set of genes differentially expressed by IFNβ and IFNλ, and not by IFNα, correlate with an antiproliferative or apoptotic pathway, we analysed the epithelial expression profile in response to IFN exposure. 363 genes are induced in response to any of the three IFNs (Figure 42A). However, we see a much greater transcriptional response by IFNλ treated AECs (Figure 42A, B). To identify the top disease pathways represented by this gene set, the 363 genes were analysed by Ingenuity Pathway Analysis (IPA). Many of the top disease pathways induced are as expected, such as 'Infectious disease' and 'Immunological disease', since IFNs play a pivotal role in the immunological response (Figure 42C). Of interest however, is the appearance of 'Tissue morphology' and 'Cell Death and Survival' within the top disease pathways induced. As the bar chart does not show how these pathways are affected, a heat map of the 'Cell Death and Survival' pathway was created (Figure 42D). Intriguingly, here we see increased induction of apoptosis, cell death, necrosis, cytotoxicity (Figure 42D). The gene set associated with this increased cell death pathway (Figure 42D) was extracted and is shown in the Appendix, Table 7. Further analysis of the pathways induced will be undertaken to identify which induced genes are central in the anti-proliferative and/or pro-apoptotic effects of IFNs on growing epithelia.

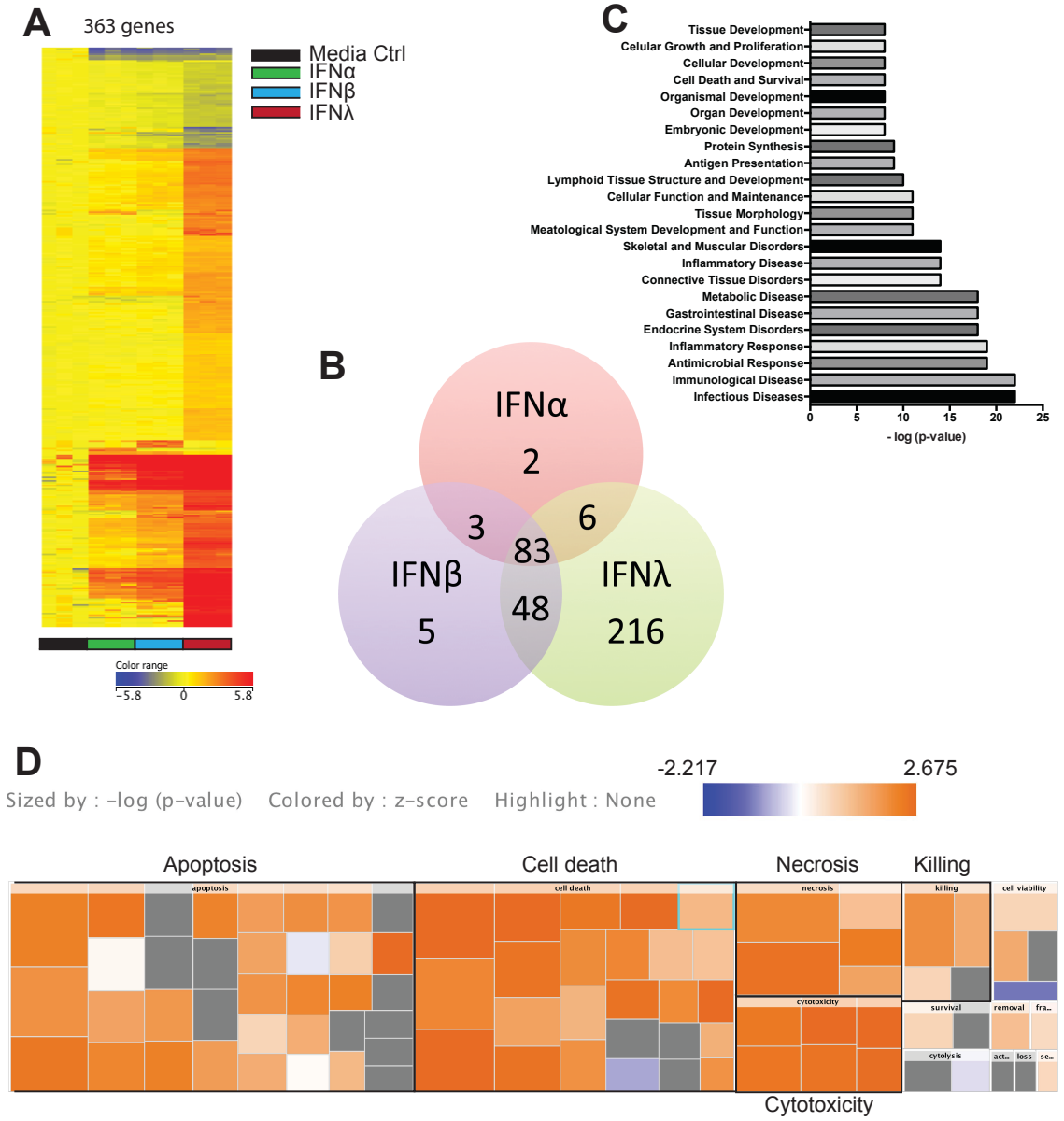


Figure 42 IFN addition to regenerating epithelia induces many anti-pathogen pathways, but can also induces cell death and apoptosis pathways

Re-plated AECs were treated with IFNα (90 U/ml), IFNβ (90 U/ml), IFNλ (90 ng/ml) or Media Ctrl from d0 of re-plating for 48 hours. RNA was collected for global analysis by Illumina.SingleColor.Mouse WG-6_V2_0_R0_1127 microarrays. Samples (n=3) were normalized to the median of the Media Ctrl group and filtered for a fold change of 2, generating 363 genes differentially expressed (A). A Venn diagram was generated using the gene-set from A to show the number of genes most differentially expressed by IFNα, IFNβ or IFNλ (B). The top disease pathways associated with the 363 gene-set were analysed by Ingenuity Pathway Analysis (C). Cell death and survival was amongst the top pathways induced. A heatmap showing the subcategories within this pathway and the IFN effect on them is displayed (D) One way

Given that we observe an antiproliferative effect of IFNβ and IFNλ, and not of IFNα, we hypothesised that the gene set differentially expressed by the combination of IFNβ and IFNλ rather than the set induced by IFNα would be inducing the apoptotic pathways seen in Figure 42. We therefore examined the whole gene set induced by IFNα and analysed these 94 genes by IPA (Figure 43A). Again we see that immunological pathways are amongst the top disease pathways induced by IFNα, as expected (Figure 43B). We did not observe the induction of the 'Cell Death and Survival' pathway. Therefore, we analysed the heat map of the cell death and survival pathway and as expected we did not observe the great inductions in apoptosis and cell death as was the case in Figure 42D (Figure 43C).

As we expected that the gene set commonly expressed by IFNβ and IFNλ is inducing the apoptotic pathways, we analysed the 48 genes induced, by IPA also (Figure 44A). Many of the top disease pathways (Figure 44B) are unsurprising, and again we did not find the induction of the 'Cell death and Survival' pathway. The heat map for this pathway surprisingly did not show a great effect on apoptosis or the other pathways, which could be due to the low number of genes analysed (Figure 44C).

ANOVA, $P<0.01$, Benjamini-Hochberg multiple test correction was carried out. *Samples were analysed under the supervision of Dr Stefania Crotta.*

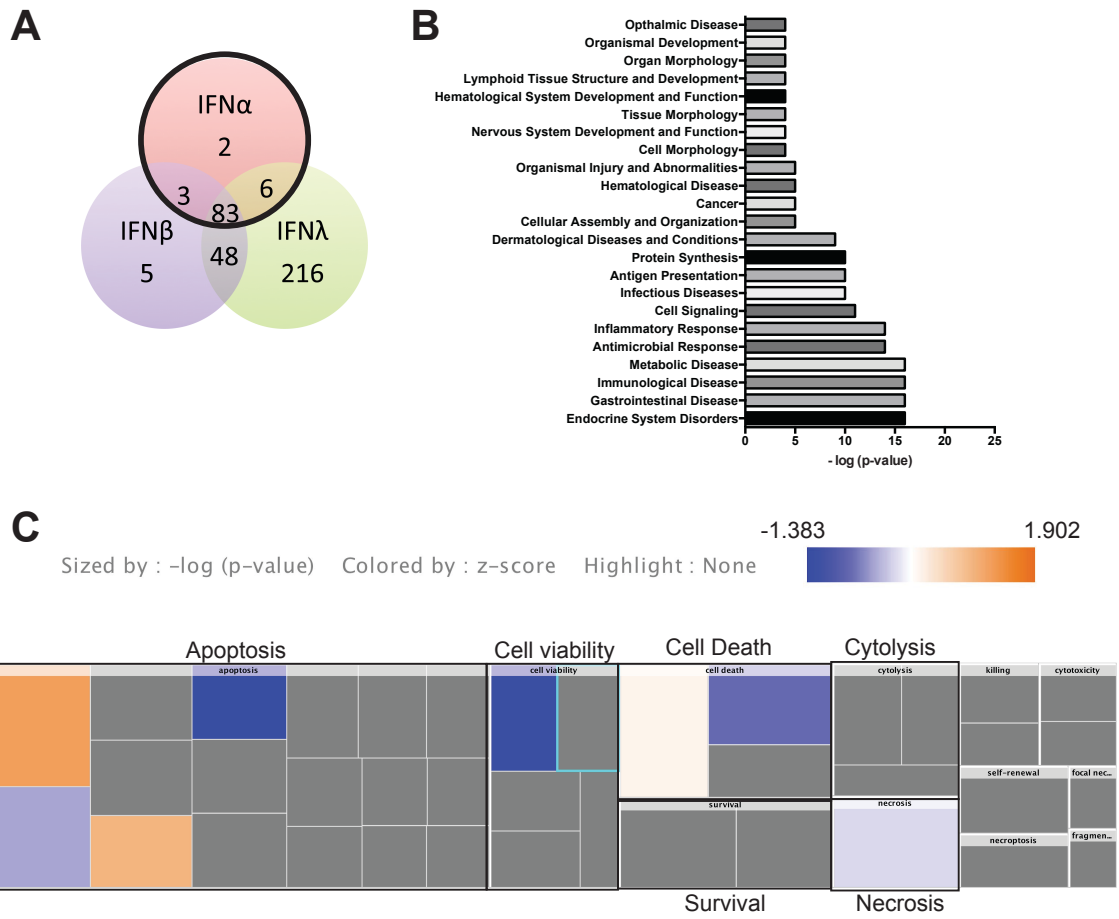


Figure 43 The genes induced by IFNα do not induce the cell death and apoptosis pathways

The Venn diagram from Figure 42 was used to generate the list of genes induced by IFNα (A). The 94 genes induced by IFNα was analysed by Ingenuity Pathway Analysis to determine the top disease pathways (B). The heatmap of the 'Cell death and Survival pathway' is displayed (C).

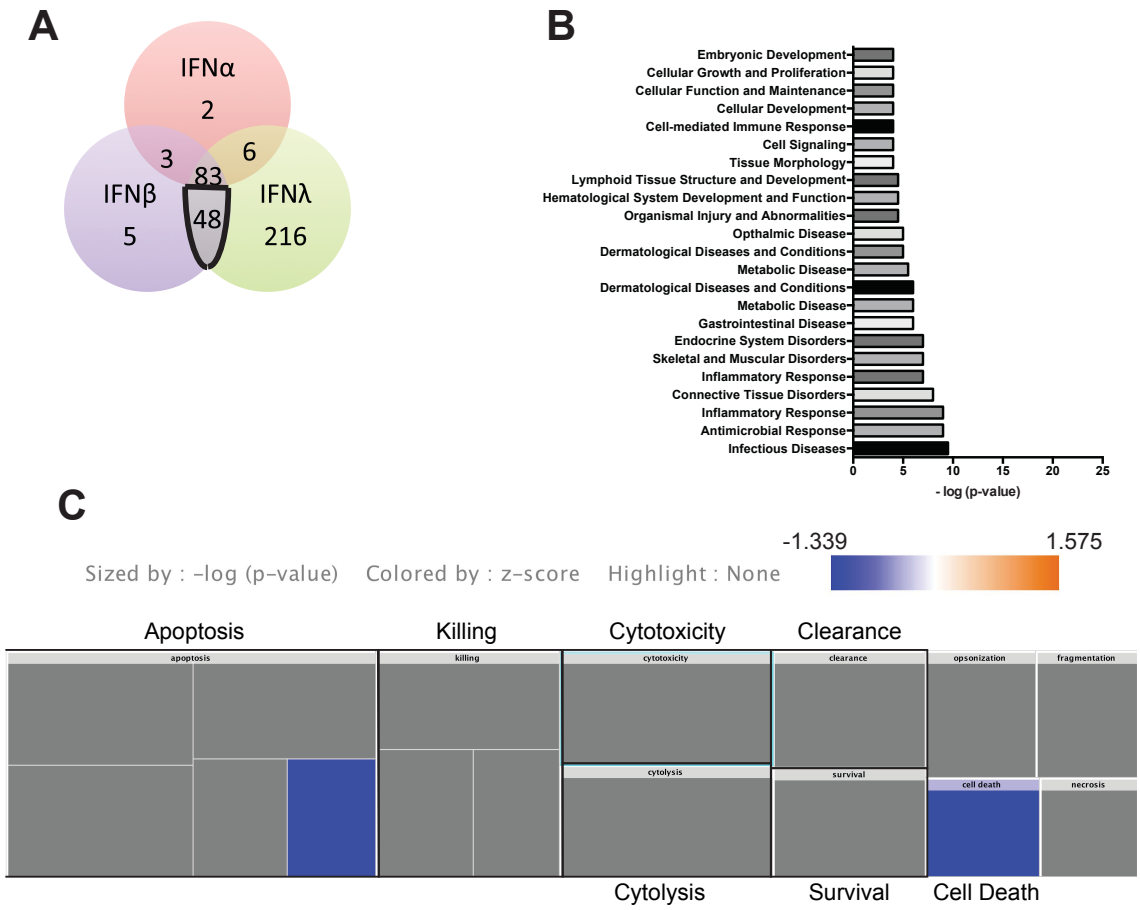


Figure 44 The common genes differentially expressed by IFNβ and IFNλ do not induce cell death and apoptosis pathways

The Venn diagram from Figure 42 was used to generate the list of genes commonly induced by IFNβ and IFNλ (A). The 48 genes induced by IFNβ and IFNλ was analysed by Ingenuity Pathway Analysis to determine the top disease pathways (B). The heatmap of the 'Cell death and Survival pathway' is displayed (C).

Although the whole gene set induced by any of the three IFNs does associate with an apoptotic pathway within these cells, we do not find that the common gene set between IFNβ and IFNλ is responsible for this effect. In an alternative analysis, we therefore compared the full gene sets induced by IFNα, IFNβ and IFNλ separately and assessed the top disease pathways induced using IPA (Figure 45). Many of the top pathways, like before, are unsurprising since IFNs are known to induce an ‘Antiviral response’ and ‘Antimicrobial response’. However, we see ‘Cell death’, ‘Necrosis’, ‘Organismal death’, and ‘Proliferation of cells’ are affected by these IFNs. In fact, we can clearly see that in general IFNλ is having the greatest effect on these pathways, with IFNα having little to no effect. This confirms our previous findings of a greater antiproliferative effect of IFNλ. More in-depth analysis of the transcriptional profiles obtained will be required to understand by which mechanisms IFNβ and IFNλ block proliferation and differentiation.

Figure 45 The top disease pathways induced by IFNα, IFNβ and IFNλ separately

The total gene sets of IFNα, IFNβ and IFNλ treatment from Figure 42 were separately analysed and compared for the top disease pathways using Ingenuity Pathway Analysis.

6.3.3 Blockade of EGFR by IFNβ and IFNλ

Given that we do not find the induction of an apoptotic pathway by the gene set differentially expressed commonly by IFNβ and IFNλ, we postulated that these IFNs may be antagonising a pathway required for these AECs to grow. As EGF is essential for the growth of AECs (Selman 2006) and is part of the culture medium, the addition of IFN could potentially block this signalling pathway. Zoon and colleagues showed that IFNα treatment of MDCK cells reduced the ability of EGF to bind to its receptor, EGFR (Zoon *et al.* 1986). WT C57BL/6 and *Ifnar-/-* cultures were grown and starved of EGF, then re-exposed to EGF in the presence or absence of IFNα, IFNβ or IFNλ (Figure 46). In the WT AECs, EGF addition alone induces phosphorylation of the EGFR as expected. This is unaffected by IFNα. However, both IFNβ and IFNλ significantly block the phosphorylation of EGFR (p-EGFR) (Figure 46A, C). Total EGFR protein (T-EGFR) also appears to be reduced by IFNβ and IFNλ (Figure 46A, D). Quantifying the fold change of p-EGFR over T-EGFR shows that even though the T-EGFR is reduced by IFNβ and IFNλ, only IFNλ induces a significant reduction of p-EGFR (Figure 46E). In the *Ifnar-/-* AECs, this block in phosphorylation of EGFR by IFNβ is not observed, as expected. Interestingly we did not see a reduction in phosphorylation of EGFR by IFNλ addition either, which is unexpected (Figure 46B, C). In fact the phosphorylation of EGFR appears to be increased by IFNλ, although this is not significant. T-EGFR is also not affected by any IFN in the *Ifnar-/-* (Figure 46C, D), and also no significant changes are observed when the fold change of p-EGFR over T-EGFR is analysed (Figure 46E). These results suggest that IFNβ and to a greater extent IFNλ, block the phosphorylation of EGFR, thus preventing the epithelia from proliferating. These results also suggest that IFNλ acts through the IFNαβR, maybe by inducing

type I IFNs or another downstream factor which is signalling through the type I receptor. Further studies will allow us to understand better the requirement of IFNαβR for IFNλ suppression of epithelial cell proliferation.

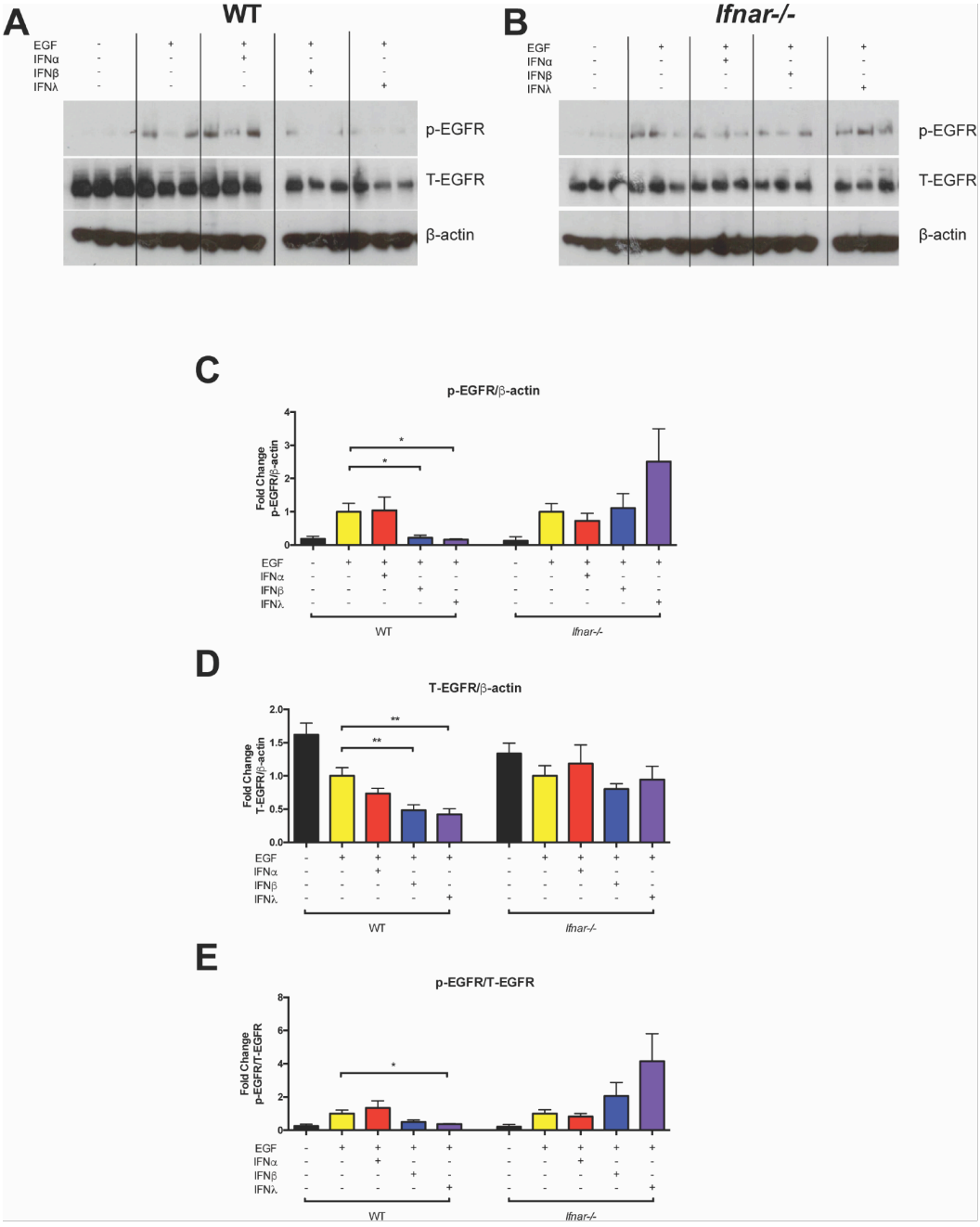


Figure 46 IFNβ and IFNλ block EGFR phosphorylation, which does not occur in *Ifnar-/-* AECs

AECs from WT and *Ifnar-/-* mice were starved of EGF on d4 post ALI for 24 hours, and then treated with equivalent doses of IFNα, IFNβ, IFNλ for 2 hours, then with EGF for another 2 hours. Samples were collected from for the detection of p-EGFR, T-EGFR and β-actin proteins by western blot. A representative blot from WT (A) and *Ifnar-/-* (B). A and B show 3 biological repeats per condition. The fold change in p-EGFR/β-actin (C), T-EGFR/β-actin (D) and p-EGFR/T-EGFR (E) was analysed. Graph show means ± SEM. Significance was assessed by unpaired t tests where * P<0.05, ** P<0.01. Data representative of 2 independent experiments, n=5 (C-E).

6.4 Conclusions and Discussion

Following lung damage induced during IAV infection, the airway epithelium must regenerate to return to homeostasis. However, cytokines present within the lung following IAV infection, such as IFNs, may affect this regeneration process. A hallmark of lung disease is the remodelling of the lung leading to goblet cell metaplasia (Fahy and Dickey 2010; Danahay *et al.* 2015). Here we have shown *in vitro* that IFNs, a family of cytokines induced by IAV infection, can reduce the overall cell numbers and number of basal cells within the epithelia, and induce pro-apoptotic pathways. We need to determine however if these lower cell numbers are due to decreased proliferation or increased apoptosis. IFNs also promote remodelling during regeneration of the airway epithelia by decreasing the differentiation of the basal cells. Our data suggest that IFNs may mediate these effects through blocking the epidermal growth factor pathway.

IFNα has been extensively used as an antiproliferative agent against tumours (Talpaz *et al.* 1987; Kikawa *et al.* 1993; Krown *et al.* 2002; Sabel and Sondak 2003; Guilhot *et al.* 2004; Aurora and Winter 2006). However, in our system IFNα did not affect basal cell numbers and did not block the phosphorylation of EGFR, unlike IFNβ and IFNλ. We utilized IFNα4 for this study, which is only one of eleven IFNα subtypes. It remains possible that we may not be using the right IFNα here, and other subtypes of IFNα should be used to see if they induce this effect. Other studies also found that IFNα did not downregulate EGFR, but rather enhanced its expression (Caraglia *et al.* 1995). We did not observe an increase of receptor levels, but this could be due to Caraglia and colleagues utilizing a human oropharyngeal epidermoid carcinoma KB cell line that may be responding to IFNα differently. Caraglia *et al.* also do not specify which subtype of

IFNα they used in these studies. EGF addition although was found to exacerbate IFNα induced barrier disruption of LLC-PK1 porcine proximal tubular cells (Lechner *et al.* 2007). IFNβ however did elicit a greater antiproliferative effect than IFNα in regenerating AECs by significantly reducing the basal cell number and blocking EGFR phosphorylation. IFNβ was also seen to induce a 'Cell death' pathway within the regenerating epithelial, whereas again IFNα did not. These differing effects between the type I IFNs could be due to affinity for their corresponding receptors. IFNβ was found to have an approximately 50 fold higher affinity for IFNAR than IFNα2 (Lamken *et al.* 2004; Gavutis *et al.* 2005). Another study linked this higher affinity to increased antiproliferative effects (Jaitin *et al.* 2006). Type I IFNs are known to disrupt the cell cycle thus exerting their antiproliferative effect. Although recently, another mechanism has been proposed whereby type I IFNs activation of β-catenin lead to reduced proliferation of intestinal epithelial cells (Katlinskaya *et al.* 2016). The blocking effect of type I IFNs on the EGFR pathway has to our knowledge not been shown before.

The interest into the antiproliferative effect of IFNλ has gained momentum since its discovery in 2002 (Kotenko *et al.* 2002; Brand 2005; Maher *et al.* 2014). Since its discovery is more recent than that of type I IFNs, little is known about the antiproliferative mechanisms induced by IFNλ. Of the three IFNs, IFNλ induced the greatest antiproliferative effect on the basal cells, and induced more strongly the 'Cell death' pathway as defined by IPA. The R&D IFNλ solution we used for this Chapter includes IFNλ3 and IFNλ2. Interestingly, IFNλ3 has been shown in the literature to induce greater expression of ISGs than IFNα following viral stimulation (Egli *et al.* 2014). A number of explanations have been put forward to account for this reported difference, including transcription factor differences; the presence of

SNPs regulating IFNλ expression or activity; receptor expression regulation; receptor binding affinity and feedback loops (Egli *et al.* 2014). A greater antiproliferative effect of IFNλ1 over IFNα-2a was found on a human keratinocyte cell line HaCaT by Maher and colleagues, who attributed this effect to prolonged JAK/STAT signalling (Maher *et al.* 2014).

In our transcriptional analysis, we observed more genes differentially expressed by IFNλ than the other IFNs, in the face of a careful titration performed initially. This discrepancy needs to be resolved and may have to do with the fact that the initial titration was done on cultures grown to confluence from tracheal cells *ex vivo*, whereas the expression profiling was performed on expanding cultures started by replating low numbers of mTEC cells (as explained in Figure 39A versus E), to have a predictable and high content of growing basal cells. These latter culture conditions may lead to a higher IFNλ responsiveness, which may happen during proliferation of basal cells, or basal cells per se are more responsive to IFNλ than more differentiated cells. More work is needed to understand if IFNλ responsiveness differs between airway epithelial cell types or within basal cells during expansion versus confluence.

We found IFNλ and IFNβ, but not IFNα, blocked phosphorylation of EGFR and total EGFR protein. There is conflicting reports on the action of type I IFNs on EGFR expression, but the effect of IFNλ on EGFR expression has not yet been studied (Martyré *et al.* 1990; Eisenkraft *et al.* 1991; Scambia *et al.* 1991; Caraglia *et al.* 1995; Yang *et al.* 2004). EGF and EGFR are required to regulate epithelial repair and therefore IFN blockade of this signalling pathway would be detrimental to repairing epithelia (Selman *et al.* 2008). IFNα or IFNβ treatment of *Ifnar-/-* AECs did not block the phosphorylation of EGFR. Interestingly, when the *Ifnar-/-* AECs were

treated with IFNs and tested for phosphorylation of the EGFR, no IFN type induced this blockade. This would imply that IFNλ mediates its effect through the type I receptor. To ensure that this is indeed the case, AECs from IL28R-/- and *Ifnar*/IL28R-/- AEC should also be utilized. Furthermore, these four genotypes of AECs should be assessed for their ability to reach confluence and the effect on the different cell subsets following growth with IFNs. One study which ablated *Ifnar1-/-* specifically in the intestinal epithelial cells, found an increase in proliferating cells (Tschurtschenthaler *et al.* 2014), which along with our results suggests that type I IFN antagonizes epithelial growth and is potentially detrimental during epithelial wound healing. Overall, our results indicate that IFNβ and IFNλ can prevent wound healing through a blockade of EGF signalling.

EGF is just one member of the epithelial repair family. Amphiregulin, transforming growth factor-α (TGFα), betacellulin, heparin-binding like EGF-factor, and epiregulin (EREG) are all ligands for EGFR (Yarom and Jonker 2011). Interestingly, the set of genes induced by any IFN treatment contains the gene for FGFR, which was downregulated. This is interesting as FGFR2b is expressed on epithelial cells and Fgf10, which signals through FGFR2b, can counteract IAV-induced repair failure to restore barrier function in the lower lung (Igarashi *et al.* 1998; Braun *et al.* 2004; Quantius *et al.* 2016). NGFR, a growth factor receptor, is also clearly downregulated by IFNβ and IFNλ as seen by flow cytometric analysis. These results may therefore highlight that IFN treatment not only blocks EGFR signalling, but can also potentially block other growth factor receptors. Blockade of EGFR has been found to induce increased secretion of amphiregulin and TGFα in colorectal cancer cells, protecting cells from the effect of EGFR blockade (Hobor *et al.* 2014). GM-CSF and IL-22 have also been identified as contributing to lung

repair following IAV induced damage (Wolk *et al.* 2004; Pociask *et al.* 2013; Rösler and Herold 2016). The effect of IFNs on these ligands, and vice versa whether addition of these ligands abolishes the antiproliferative IFN effects, should be determined.

Not only can IFNs be antiproliferative and pro-apoptotic, but they may also drive remodelling of epithelium. This remodelling of the lung is common in many airway diseases as discussed in Chapter 4. We showed that IFNα, IFNβ and IFNλ had the ability to reduce differentiation of the cells, with IFNλ having the greatest effect and IFNα having the least. IFNα and IFNλ were found previously to reduce ciliated cells and increase goblet cells in human lung airway epithelia cells (Danahay *et al.* 2015), although IFNβ was not assessed in this system. This paper, unlike our results, did not find a discernable difference between the effects of IFNα or IFNλ on goblet or ciliated cells, nor did it detect any effect on the basal cells. Danahay and colleagues treated fully-grown human 3D epithelial organoid cultures and therefore the cultures may not be proliferating greatly, thus reducing the effect of the IFNs on the basal cells. Our flow cytometric analysis panel cannot distinguish between ciliated and goblet cells and so we do not know the effect on goblet cells and whether they are indeed increased here as they are in Danahay *et al.*, therefore immunofluorescence staining for goblet cells or PCR analysis of goblet cell marker expression in this system would be helpful.

For the differentiation to ciliated cells aryl hydrocarbon receptor (AhR), a transcription factor best known for mediating the effects of toxins, signalling is required (Villa *et al.* 2016), and Notch signalling must be blocked (Boon *et al.* 2014). IFN may be antagonising or enhancing these signalling pathways respectively. In fact, interaction between AhR and type I IFNs has been previously described,

whereby AIP (aryl hydrocarbon receptor interacting protein) was found to be a new binding partner of IRF7, a key inducer of type I (Zhou *et al.* 2015) and III IFN expression (Osterlund *et al.* 2007). Administration of IFNβ was found to suppress inflammation within the central nervous system through activation of AhR (Rothhammer *et al.* 2016). In the context of a viral infection however, AhR signalling negatively regulated the type I IFN driven antiviral response (Yamada *et al.* 2016). Binding of the AhR and IRF7 proteins could potentially inhibit the induction of type I and III IFNs and thereby prevent the ciliopathy state induced by these IFNs. IFNα was found to block Notch signalling in human bone marrow mesenchymal stem cells (Su *et al.* 2015). Although this is a different cell type that is not present in the mTEC cultures, it may indicate why IFNα induces the least effect on ciliated cells. However, we do not know if IFNβ and IFNλ can initiate the same block on Notch signalling. Furthermore, increasing the concentration of IFNα does have an effect on ciliated cells, which may indicate that if the Su *et al.* study does recapitulate in mTEC cultures, higher concentrations of IFNα may supersede this blockade.

Whether these effects would still be seen *in vivo* is unknown. To do this we first need to set up a model of reproducible lung damage. IAV infection induces lung damage but is itself a strong IFN inducer, and thus does not allow for the modification of IFN levels independently from damage induction. Naphthalene treatment of mice would be a better way to induce reproducible lung damage. Naphthalene is a chemical which when metabolized ablates club cells so that ciliated cells squamate and cover the basement membrane. The lung epithelium must then repair by basal cell proliferation and differentiation. IFN addition to naphthalene-induced lung damage could not yet be performed. It is hypothesised

that naphthalene will induce damage within the lung, and IFNα will have little effect on repair, whereas IFNβ and IFNλ will impair or fully prevent regeneration of the lung, resulting in persistent damage. Furthermore, if the epithelia do repair, IFNβ and IFNλ could induce a remodelling of the lung resulting in decreased ciliated cells, creating an altered cellular composition of the epithelium, which may render the host more susceptible to subsequent infection, since there are fewer cilia to guarantee mucociliary clearance.

Overall, we have shown that the presence of IFNβ and IFNλ, but not IFNα, during epithelial repairing prevents proliferation of basal cells, induces a 'Cell death' pathway, and inhibits differentiation of AECs. We need to determine whether this is an antiproliferative or a pro-apoptotic effect and suggest that IFNβ and IFNλ mediate this effect through the prevention of EGFR signalling. There are indications that FGFR and NGFR are also downregulated in response to IFNs. All three IFNs can however induce a remodelled state of the AECs, if the epithelia have enough basal cells to grow, resulting in fewer differentiated cells. We observed a striking effect of IFN addition in the reduction of ciliated cells. Finally, we found that IFNλ induces a greater effect on each of these outcomes. Many fatalities from severe IAV infection have been associated with extensive pathology and epithelial damage (Herold *et al.* 2008; Mauad *et al.* 2010). While much attention is given immune-mediated damage, we propose here that IFNs produced during viral infection could be preventing the lungs from repairing, thus contributing to the increased mortality. Furthermore, should the host repair the epithelium, the IFNs present during this process may have contributed to 'remodelling' of the epithelium, resulting in a diseased-prone state, which could potentially lead to susceptibility to other invading pathogens.

Chapter 7. Conclusions

This thesis assessed in depth the response of AECs when exposed to different pathogens or immune stimuli. We investigated the contribution of AEC responses to severe disease caused by IAV-*S. pneumoniae* co-stimulation. We compared AEC responses and cell composition between IAV-resistant and susceptible inbred mouse strains. We studied how the restriction of IFNλR expression to AECs allows for a better treatment of influenza by IFNλ than by IFNα. Finally, we assessed how AEC repair and composition can be significantly affected by IAV-induced IFNs.

We studied the response of the upper airway epithelial cells in these different scenarios and in some cases did not find what we hypothesized. This could be due to the lack of other cytokines and immune cells within the *in vitro* system. To better reflect the environment of AECs *in vivo*, co-culture systems with immune cells should be set up to study the different hypothesises posed in Chapters 3, 4 and 6. However, a co-culture system is not without its own complications. Deciphering which immune cell is responding, or whether the response is initiated by the AECs, or by the innate immune cells becomes a challenge as it would require re-separating cells prior to analysis. Also, owing to the position of the innate immune cells compared to the AECs *in vivo* (i.e. on the airway luminal side, which corresponds to the liquid free upper transwell chamber, or the stromal side, which corresponds to the lower, culture-medium filled transwell chamber) makes the set up difficult.

The mTEC culture is a potent experimental system to study the response of the upper airway epithelium in isolation, however it too comes with limitations, as the cultures can be difficult to grow and can often fail. As the 129S8 AECs show a reduction in ciliated cells that is not found *in vivo* in 129 tracheas during steady

state, suggests that plating these cultures may lead to culture not fully reflecting the *in vivo* situation. Effects seen *in vitro* should always be confirmed *in vivo*, however the massive responses observed *in vitro* may be diluted when taken *in vivo.* The mTEC system we employed also only represents one type of epithelium: the upper airway epithelium. Some disease processes may happen in other lung cell types such as endothelia or alveolar epithelia. In fact Teijaro *et al.* suggest it is the endothelial cells that produce the bulk of IFNs following IAV infection and are responsible for the cytokine storm found in severe influenza (Teijaro *et al.* 2011). This may explain why we do not find high type I or III IFNs from the 129S8 AECs. Since primary influenza infection is an upper respiratory tract infection, we do think that mTEC cultures are the most appropriate primary cell model to study influenza-related responses.

In Chapter 3 we showed that airway epithelia do respond to both viral and bacterial stimulation. Our main hypothesis was that epithelial responses may contribute to the severe phenotype of lung damage, high cytokine responses and bacterial spread observed in co-infection. We first asked if the antimicrobial response by AECs was impaired by the previous virus or IFN exposure. We found little to no induction of AMPs by bacterial stimulation alone, which was generally not affected by co-stimulation. As we did not see an induction of AMPs, we did not pursue this hypothesis further. Future studies could however quantify many more AMPs and test bactericidal activity of epithelial supernatants in functional assays. We next asked if AECs contribute massively to the high proinflammatory cytokine and chemokine signature observed *in vivo* in response to the combined viral-bacterial stimulus. We found that the viral stimulation alone drove the response of IFNs, ISGs, and the overall transcriptional response from AECs, which was only

slightly changed following co-stimulation. We did however see an increased proinflammatory cytokine and chemokine induction from the AECs during co-stimulation in comparison to each single stimulus. Future chemotaxis assays could quantify how immune cell recruitment is affected following co-stimulation. Finally, we asked whether increased epithelial damage and death is induced by the combined stimulus. Much like the AMPs, we found that apoptosis related receptors were not induced following either single stimulus nor induced in co-stimulation, and the monitoring of mTEC cultures during co-exposure did not indicate major cell death occurring. This was however not formally measured by apoptosis assays or measurements of epithelial integrity. Overall, we found that the *in vitro* AEC cultures do not closely reflect the effects of co-infection we observe in the lung *in vivo*, however they appear to be contributing to the documented massive increase in inflammation and immune cell recruitment during co-infection.

While this study was being undertaken, a parallel study was being performed *in vivo* within our lab. It was found that one of the drivers of the high cytokine response was the massive expansion of bacteria (Ellis *et al.* 2015). It is therefore unlikely that an increased epithelial response to co-stimulus alone explains the massively increased cytokine and chemokine production, but rather, that higher bacterial numbers *in vivo* are a main driver of this.

We did not see an induction or suppression of AMPs during IAV infection that could in part explain this massive expansion of bacteria seen *in vivo*. The *in vivo* model however was carried out on specific pathogen free mice and therefore the airways are not free from bacterial colonisation. There is the possibility that it is not just *S. pneumoniae* that is disseminating during co-infection *in vivo,* and the presence of these other bacteria may be contributing to the overall response. The

lung flora present at steady state within these mice may also have a priming effect on the airway epithelia that gets lost in sterile cell culture conditions. This may explain aspects of the reduced responsiveness of mTEC cultures. Furthermore, the lack of innate immune cells in the mTEC culture could potentially be another reason for this. Innate immune cells can produce IL-22, a member of the IL-10 cytokine family, which can bind to epithelial cells to induce many AMPs, and can augment the expression of S100A8 and S100A9 (Wolk *et al.* 2004; Liang *et al.* 2006). These innate immune cells may also be primed by the lung flora at steady state to produce IL-22. However, IL-22 treatment of the epithelial cells alone did not induce a great response (data not shown), which may indicate that another cytokine is missing. This could potentially be IL-17 as Liang *et al.* showed that IL-22 and IL-17 synergised in the production of AMPs (Liang *et al.* 2006), or the combination of IL-22 signals with TLR-mediated signals may be required for AMP induction. A co-culture system would allow us to determine the impact of the innate immune cells to the AMP response from the AECs.

Results from the lab also showed that the immune-mediated damage was crucial for severity during co-infection. Although we did not find an increase in apoptosis related genes, this could also be due to the lack of the innate immune cells. pDCs are the main producer of type I IFN which is known to upregulate death receptors on the surface of AECs. Co-cultures would therefore allow us to determine if in the presence of innate immune cells, apoptosis related receptors are induced.

If we look at the results obtained in Chapter 3 in the context of the findings from the thesis as a whole, a range of possible scenarios can be envisaged. A study by Kash *et al.* found a loss in epithelial cell proliferation and lung repair

mechanisms following co-infection (Kash *et al.* 2011). This is reminiscent of the results from Chapter 4 and 6. Therefore one could use the mTEC system to specifically determine if this loss in epithelial repair function in co-infection is orchestrated by the epithelium alone. One could measure epithelial permeability or epithelial repair after injuring the epithelia by scratch, or after replating low numbers of basal cells in co-exposure conditions, to understand if combined viral and bacterial stimuli can impair epithelial repair directly. The findings from Chapter 6 demonstrate that in the presence of high IFNβ and IFNλ proliferation is hindered, leading to reduced repair of the epithelium. Development of differentiated cell subsets is also affected, leading to an altered epithelial cell composition. Any deviation from normal cell ratios within the lung epithelia can greatly impact the mucociliary action in the airways, hindering the ability to remove any pathogens. This may explain the increased epithelial damage and outgrowth of bacteria observed during co-infection.

Kash *et al.* also showed a reduction in the epithelial repair factors HGF and FGF levels during co-infection (Kash *et al.* 2011). In Chapter 6, we describe the blocking effect of IFNβ and IFNλ on EGF signalling. Therefore, it would be interesting to determine if the reduction of HGF and FGF during co-infection is due to these IFNs. Blocking IFNs *in vivo* could be useful for the prevention of invasion of the bacteria. However, there are a number of caveats with this since the timing of antiviral vs. antiproliferative effect, and bacterial colonization are unknown. Blocking IFNs too early could be detrimental for the antiviral response of the immune system, leaving the host vulnerable to an unchecked IAV infection. Blocking too late may be superfluous as the damage has already occurred and bacteria have already disseminated.

IFNs were not the only cytokine reported to cause a remodelling effect. Danahay and colleagues reported a variety of cytokines which had an effect on the different subsets of the AECs (Danahay *et al.* 2015). This remodelled epithelium can result in ciliopathies or goblet cell metaplasia, which affects the proper mucociliary action of the lung. Many of these cytokines have not been tested for their antiproliferative abilities. Danahay *et al.* did not show an effect on basal cells by IFNα or IFNλ, yet in Chapter 6 the antiproliferative effect of IFNλ is quite striking. Owing to the massive proinflammatory cytokine signature in co-infected mouse lungs, there is the possibility that some of these cytokines are also affecting the repair of the lung epithelium, thus further increasing epithelial damage, epithelial remodelling and augmenting morbidity and mortality. Therefore, testing the antiproliferative effect of the described cytokines, and a better understanding of IFN effects on secretory cell differentiation, might also be interesting.

In Chapter 4, we hypothesized that genetic differences at the level of the airway epithelium can determine the outcome of disease. We first asked if the high IFN response by IAV-susceptible mice, 129S8, was orchestrated or reflected by the airway epithelium in response to IAV. We showed that epithelial responses *in vitro* did not recapitulate the *in vivo* situation. 129S8 AECs induced little to no IFN in response to IAV infection. This may be due to the lack of innate immune cells within the culture. pDCs were found to be the main producer of IFN during IAV infection *in vivo* (Davidson *et al.* 2014). pDCs induce IFNs via a TLR7 pathway, whereas the AECs respond predominately via the RIG-I pathway (Diebold 2004; Lund *et al.* 2004; Iwasaki and Pillai 2014). These different recognition pathways may explain why 129 pDCs produce much higher IFN amounts upon IAV exposure than B6 pDCs, while epithelial cells don't. We next asked if it was epithelial

response depended on the responsiveness to IFN and not the induction of it. We showed that 129S8 AECs had an increased responsiveness to IFN or IAV infection as measured by induction of ISGs and proinflammatory cytokines. However, we did not see any induction of death receptors following IAV infection. Again this could be explained by insufficient amounts of IFN induced by the epithelium itself and the lack of innate immune cells which can produce IFN in the culture system. It is also possible that other cytokines that are required for the induction of death receptors are missing from the *in vitro* culture system. Interestingly, the media control of the 129S8 AECs had a higher resting state level of DR5 and FAS. Finally, we asked if the 129S8 epithelium had a reduced potential to repair following injury. We found that this was indeed the case. Additionally, the 129S8 AECs showed an altered cellular composition, displaying less basal and differentiated cells, reminiscent of remodeling in chronic disease states.

Taking these findings together with other results in this thesis, it would be interesting to investigate if the higher levels of apoptosis-related receptors in the uninfected AECs is due to the regeneration process after plating, and therefore may not reflect the quiescent state *in vivo*. This apoptotic-prone state of the 129S8 also fits with the decreased ability to repair. Since 129S8 AECs are taken from a high IFN producer, and cell damage is known to induce IFNs (Yu *et al.* 2015), could there be a higher tonic level of IFNs during the growth of the AECs, thus reducing the ability to grow and affecting the different cell subsets? We did not observe any IFN production by the 129S8 at resting state, but we do see slightly higher ISG levels. This was however analysed on fully differentiated AECs and as such the IFNs potentially present during growth are unknown. Furthermore, we have shown that even in the presence of low concentrations of IFNλ, the antiproliferative effect

can still be seen, thus the IFN amounts required to induce these processes may be below the limit of detection by ELISA. This is also corroborated by the slightly higher ISG expression on steady state 129S8 AECs.

The fully differentiated 129 epithelium has reduced basal and differentiated cells compared to C57BL/6. It would be interesting to analyse if the lack of IFN production following IAV infection from these cells is due to this remodelling, thus leaving the AECs open to an unchecked IAV infection. The production of IFN was found to be somewhat decreased in AECs from patients with chronic lung diseases, such as COPD and cystic fibrosis, which have a remodelled state of the lung (Zheng *et al.* 2003; Mallia *et al.* 2011). It is unknown if the ability to produce IFNs in response to IAV infection varies between basal and differentiated cell subsets. It is also not known how long this remodelling lasts, or whether the AECs will stay remodelled. Further quantification of the cell subsets over time should therefore be undertaken. Although we showed that the different cell subsets do not appear to be affected in naïve 129S8 tracheas *ex vivo*, a more in-depth study is required to see if there are less basal and differentiated cells during steady state or even after infection *in vivo* compared to C57BL/6 mice.

Blocking IFN during 129 AECs growth/repair could prevent the antiproliferative effect and allow for normal AEC homeostasis. However, 129 *Ifnar-/-* AECs do not grow (data not shown). This may show that although high IFN levels can block growth, no IFN at all is also not conducive to growth of the AECs. The *ifnar-/-* may also have a tonic signal of IFNλ, which was shown to be the most potent IFN affecting AEC growth and differentiation. Therefore a blocking antibody against IFNλ may be helpful in this system. Alternatively, this growth defect could also be potentially due to a separate factor missing in 129 cultures, unconnected to

IFN, which is required for growth. Given these genetic differences observed between C57BL/6 mice and 129S8 mice, the 129S8 mice could potentially be more susceptible to not only primary IAV infection but also to co-infection. Utilising the 129S8 epithelium may therefore indicate how an individual with a remodelled lung, such as in severe asthma, cystic fibrosis, or COPD, are more susceptible to co-infections (Wark *et al.* 2013), thus allowing for better understanding and possible treatments.

In Chapter 5, we hypothesized and confirmed that the restriction of IFNλ responsiveness and productive IAV replication to AECs allows IFNλ to limit IAV spread through antiviral gene induction in the relevant cells, without over stimulating the immune system and driving immunopathology as seen with IFNα. We therefore propose IFNλ as a superior treatment option for human IAV infection.

Along with the experiments proposed in Chapter 5, it would be of interest to stain for Mx1 protein in the lung, as has been elegantly shown in the intestinal tract of mice following treatment with IFNα and IFNλ (Mahlakoiv *et al.* 2015). Staining for Mx1 in the gut allowed Mahlakoiv *et al.* to show a compartmentalised IFN system, where the epithelium respond primarily to IFNλ, and other cell types require IFNαβ for induction of their antiviral defence. Staining of lung sections after IFNα or IFNλ treatment may allow us to identify if the immune cells are responding to IFNλ.

Although we utilised both mouse and human cells, a translation of this study into ferrets would provide a better confirmation of the usefulness of IFNλ as a treatment option, as ferrets are considered a more clinically relevant model of IAV due to the distribution of sialic acid species on their lung epithelia which more closely reflects that of human AECs (Jayaraman *et al.* 2012). The ability of ferrets

to transmit the virus from one another could also theoretically highlight the effectiveness of IFNλ in preventing transmission (Herlocher *et al.* 2001).

IFNλ treatment reduces IAV-induced apoptosis of the AECs (Davidson *et al.* 2016). However our results in Chapter 6 emphasise the importance of a strict treatment regime. IFNλ treatment should be stopped before repair of the lung commences to prevent the antiproliferative and remodelling effects of IFNλ from having deleterious outcomes. Since this effect is not observed by IFNα, it could be proposed that IFNλ should be only be used as a treatment early in infection shortly after onset of symptoms, and then replaced with small doses of IFNα, low enough to not induce immunopathology, but to ensure full IAV clearance without affecting repair of the lung. Unfortunately, the exact timings of this would be difficult to determine in a clinical setting, due to the fact that time of infection is mostly difficult to determine when patients present with their symptoms. Furthermore, given the increased AEC apoptosis following IAV in 129S8 mice (Davidson *et al.* 2014), and a lower ability to repair, the exogenous IFNλ may be exacerbate this apoptosis and reduce repair in patients that are represented by the 129S8 type of response to influenza. Since *in vitro*, 129S8 mTECs show changes in cellular composition that are reminiscent of those observed in human lung diseases, further careful study is required to elucidate how IFNλ treatment will affect the AECs of such patients.

The IFN-linked susceptibility difference between C57BL/6 and 129S8 mice may be due to timing of IFN induction rather than the total IFN amounts, as 129S8 mice have prolonged IFN during IAV infection (Davidson *et al.* 2014). This prolonged IFN production in 129 mice could therefore hinder repair. To decipher if this is the case, treating IAV infected mice with IFNα or IFNλ only early or only late

during infection mice may allow a better understanding of the effect of mistimed IFN production clashing with repair processes.

In Chapter 6, we hypothesised that the antiproliferative effects of IFNs would inhibit repair of AECs by restricting EGF signalling. We have shown that IFNβ and IFNλ have an antiproliferative or pro-apoptotic effect on repairing AECs whereas IFNα does not. We suggest that IFNβ and IFNλ mediate this effect through the prevention of EGFR signalling. All three IFNs can induce a remodelled state of the AECs, resulting in less differentiated cells. Finally, we show that IFNλ induces a greater effect on each of these outcomes. These effects may be especially relevant in high IFN producing individuals. During IAV infection of 129 mice, there is a second peak in IFN levels around day 6 post IAV infection (Davidson *et al.* 2014), a time in which the lung epithelium may have started to repair. Presence of these IFNs may disrupt the repair process leading to increased lung leakiness and potentially mortality of the host. Therefore, it may be beneficial to block IFNs during this time to allow for the lung to repair.

These preliminary results need further work to understand the importance *in vivo* of the effects of IFNs and their mechanisms of action. We also do not yet know if IFNs specifically block only EGF and not other growth factors, such as FGF and amphiregulin, therefore these other growth factors need to be tested. GM-CSF, amphiregulin, and IL-22 have also been shown to be required for repair of the lung following IAV infection (Monticelli *et al.* 2011; Pociask *et al.* 2013; Rösler and Herold 2016). These cytokines are mostly induced by innate immune cells and therefore the mTEC system cannot fully model these effects without setting up a co-culture system or adding these cytokines to the culture. The effect of IFNs on GM-CSF and IL-22 should hence be tested *in vivo*. It is not known whether

blockade of only one of these growth factors is sufficient for prevention of repair or if a blockade in all is required *in vivo*.

Type I IFNs have been shown to activate non-epithelial cells which respond by promoting increased epithelial cell proliferation and wound repair through the induction of the ISGs Apolipoprotein L9a and b (Sun *et al.* 2015). It may therefore be possible that the antiproliferative effect of IFNβ or IFNλ could be counteracted by this immune cell-induced signal for cell proliferation. This would be very interesting, and a co-culture system here would allow for further investigation into this.

Overall, we have shown in this thesis that airway epithelial responses to bacterial-viral co-exposure reflect some of the synergistic effects found *in vivo* in co-infection, in particular with respect to increased production of proinflammatory cytokines and chemokines. The AECs can also respond differently to infections depending on the host genetic background, which may contribute to reduced ability to repair the damage caused by IAV infections. Regeneration of the AECs in these individuals may also lead to a change in the cellular makeup of the epithelium which somewhat reflects the remodelling found in chronic inflammatory lung diseases. This remodelled state may affect the function of the lung and lead to increased susceptibility to other infections. The restriction of the IFNλR to the AECs also allows for a targeted treatment against IAV with IFNλ, which would not induce immunopathology as seen with IFNα. Finally, we have shown that IFNβ and IFNλ can affect the ability of the AECs to regenerate by blocking EGF signalling. The three IFNs tested can reduce basal and differentiated cells, with IFNλ inducing the greatest effect. These results show that studying epithelial responses and repair in

different scenarios can further our understanding of disease severity in lung infection and inflammation.

Chapter 8. Appendix

RIPA Buffer	Concentration
TrisHCL	50 mM (pH 8)
NaCL	150 mM
EDTA	1 mM
EGTA	1 mM
Ne4P2O7	2.5 mM
NeF	1 mM
Ne3VO4	1 mM
Trition-X-100	1%
SDS	0.30%
Protease inhibitors	1%
Water	

Table 1 The constituents of Ripa Buffer

Antigen	Fluorochome Conjugate	Company
CD133	PerCp-eFluor® 710	eBioscience
CD49f	PE-Cyanine7	eBioscience
CD66a	APC	eBioscience
EdU	Alexa 488	Click-iT® EdU ThermoFisher
NGFR	FITC	Advanced Targeting Systems
Zombie live/dead	Violet laser	Biolegend

Table 2 Flow cytometry antibodies for mTEC stains

Antigen	Fluorochome Conjugate	Company
CD133	PerCp-eFluor® 710	eBioscience
CD45.2	Pacific blue	Biolegend
CD66a	APC	eBioscience
EdU	Alexa 488	Click-iT® EdU ThermoFisher
Ep CAM	APC-Cy7	Biolegend
Zombie live/dead	Violet laser	Biolegend

Table 3 Flow cytometry antibodies for whole lung stains

Table 4 List of the genes differentially expressed by co-exposed AECs compared to LPS or IAV exposure alone in Figure 16

Symbol	FC ([X31+LPS] vs. [mock])
4833436C18Rik	-6.616498
Itln2	-1.4508847
2900009C16Rik	-4.8684654
Gstp2	-4.061549
Kcnab2	-12.71074
D930036B08Rik	18.017847
2010001J22Rik	-63.868076
2010001J22Rik	-24.506163
Nfkbie	12.563931
Nfkbie	17.378511
Tmem171	26.628689
Ldlr	5.5615287
LOC237296	-17.377806
4930449E07Rik	-47.04908
1700019F09Rik	-36.782623
Rorc	-3.8320642
Tcte1	-25.223228
Cysltr1	-3.1573703
2810022L02Rik	32.489597
Galm	-4.668308
Plekha7	5.395251
Ifi205	21.468693
Ifi205	25.575924
Ifi205	38.706608
Ifi205	10.88376
Fhad1	-4.909529
H2-Bf	83.555016
Timp4	-10.896376
Iqca	-17.10718
A430065P05	111.68383
Purg	-1.1545076
Spag8	-7.7218614
Scgb3a2	-9.744204
Ldlr	4.163194
Plunc	-4.050612
Mpv17l	-2.3889432
0610010F05Rik	-11.703206
4930579J09Rik	-37.58956
Fxyd2	-106.14038

Aox3	-14.553607
Cat	-7.6073117
1700011C14Rik	-10.53248
Cdsn	219.21442
5930430M20Rik	-13.364306
Ehd1	4.3305926
1700001C02Rik	-48.244984
B930008I02Rik	-24.080448
LOC381916	-15.685317
Zbp1	64.08602
Gsta1	-5.635457
Gsta1	-6.7499123
Slc26a4	45.52734
BC013491	-20.588362
Galc	-2.4716873
Gsdmdc1	9.479608
Gsdmdc1	27.47724
Jundm2	3.7208228
Piga	7.6550226
LOC434484	87.84375
Tnf	90.47961
Vcam1	23.509392
Slc2a9	-2.2003636
Gm1661	-112.55427
LOC240672	14.989548
BC038167	-24.324547
Tnfrsf11b	9.097863
Zzz3	-2.6000457
Cxcl1	55.917324
F630022B06Rik	11.188072
LOC381256	-3.591229
2900016B01Rik	-19.04787
Mlf1	-44.737885
Slc9a4	13.76369
Slc9a4	8.299932
Ifi203	69.495346
4933404M02Rik	-5.480723
9030623N16Rik	135.7164
Hrc	-3.0428936
A630039F14Rik	-3.4227674
LOC380787	23.989973
Dnahc2	-45.259487
Cryz	-2.1123452

Lpxn	-13.401927
D530004J12Rik	-7.014247
Edg7	-1.6235354
6820408C15Rik	-71.78285
Ptpn8	12.074154
LOC380842	-96.52662
9330161C17Rik	-3.6476393
Pim1	17.399927
1110018J23Rik	-6.598663
4933417K04Rik	-22.079964
LOC231462	1.7637912
A830087P12Rik	-6.3359547
Ddo	-52.8597
4932415A06Rik	-9.150352
Slc22a17	-4.3561683
Casc1	-45.03372
Casc1	-4.098376
9030418K01Rik	-5.8692813
LOC331595	-49.758327
1700001O22Rik	-14.378539
C2	28.046274
4933405I11Rik	5.5617485
Slc5a8	16.147255
1700066B19Rik	10.237174
A330021E22Rik	-21.889818
A330021E22Rik	-21.9644
Ebi3	16.804497
Ints6	10.819539
1700007K13Rik	-36.75222
Rhof	11.151914
Ak7	-22.122171
B230363K08Rik	-99.97411
Card15	6.587546
Slc35c1	-2.5983336
BC029169	9.023405
Acta1	-56.889294
Lrsam1	-4.6719723
Adh1	-2.258216
2610028H24Rik	-124.77361
Gvin1	88.786835
Isg20	4.583856
5033414D02Rik	4.649564
LOC384343	26.442842

Lrpb7	-32.796
2900006K08Rik	-23.98524
A730054J21Rik	-20.708189
Sncg	-1.5793785
2210413P10Rik	-3.1469152
Ifitm6	36.36291
Adamts4	300.66315
Dnali1	-23.25053
Grip1	-2.9057631
1500015A07Rik	28.102886
4933428F06Rik	-52.27514
Cd244	3.7681665
4933430F08Rik	132.17178
Aqp11	-6.6303277
Tnfrsf19	-19.310022
LOC381457	7.9593415
C630016B22Rik	5.185207
Mapk15	-20.624105
2610028H24Rik	-110.92721
scl0003155.1_68	-2.891191
4930528G09Rik	-78.82128
Cxcl2	264.14862
E030019B06Rik	-20.972475
6430628N08Rik	-3.7217908
Ceacam1	4.4437914
Chi3l4	-8.749407
Itlnb	-1.4597262
1700016J18Rik	-59.856785
1110049B09Rik	-38.420452
D4Bwg1540e	-73.22609
Acaa2	-3.225109
Acaa2	-3.9184122
BC036961	27.2213
Rtdr1	-34.40007
Lhb	-16.639118
1700007J06Rik	-32.029785
4930534B04Rik	-7.8614287
Ttc18	-17.498028
Csta	14.027455
LOC333670	-17.393137
A430073A17Rik	6.0253515
Fn3k	-21.116127
4930438O03Rik	-3.8284912

Trim44	-1.0726924
2900052P03Rik	-7.194705
Ppp1r3c	-5.756804
Lrrc50	-66.95898
Tcea3	-13.544936
Azgp1	-13.210191
Il6	6.570271
BC010462	17.017906
Aldh3b1	-10.930594
Gp49a	79.96013
Rab36	-5.5520024
4931433A13Rik	-4.344605
Adamts9	24.222624
2810422M04Rik	-10.915428
Kif9	-23.536377
Spata17	-11.298378
Rage	-4.2983847
Rage	-15.949286
Spag6	-28.453316
Spag6	-38.839592
Sprr2f	19.87772
Serpina3g	55.48399
Osbpl6	-132.87158
1700123D08Rik	-7.3263907
LOC241084	-35.116776
Ltf	12.904063
Lrrc6	-9.372501
LOC386463	74.38535
3110040M04Rik	18.400597
LOC237250	-4.581747
Adrb2	6.7966495
LOC546840	-10.911534
D330022A01Rik	-19.868681
5730402C02Rik	-19.478422
Cd177	-3.1883702
BC028975	-20.366316
Sprr2b	-3.4636607
A230069H10Rik	-1.5219984
Frmd4b	4.5070868
1700013F07Rik	-37.376266
9930023K05Rik	95.82212
9930023K05Rik	105.071815
4930451C15Rik	-20.777159

2510015F01Rik	-10.099755
1110069O07Rik	-36.470734
Slpi	10.625102
LOC381362	-9.328591
4930442L21Rik	-29.022951
Tnfrsf1b	8.102031
Tnfrsf1b	17.41249
C330001K17Rik	-26.115128
AA467197	14.979809
Tekt1	-60.483406
Tekt1	-40.70461
Ii	101.955
4932425I24Rik	-20.70129
9030611K07Rik	9.642454
LOC215949	-5.789652
Mlh1	-11.270096
Itga2	6.9616275
Spag5	-11.966632
S100a9	99.792145
1700120B06Rik	-40.166912
Slc44a1	3.2074864
Trim40	14.164214
4930465M17Rik	-5.745584
1200009I06Rik	30.346096
C330001K17Rik	-76.19055
Zmynd10	-16.34682
Map3k5	-9.48425
D630004K10Rik	-4.734312
Csf2	6.6795254
Tsga10	-14.727856
BC018371	-6.218881
Acaa2	-3.5945487
4933409I22	-53.44357
4931415C17Rik	-10.89132
Wfdc12	52.06049
LOC381737	-10.040842
4932416A11Rik	-3.2764194
Pdgfc	-4.7821374
Mmp13	4.52152
A530060O05Rik	74.501236
Ppp1r9a	-5.6219783
4932415A06Rik	-10.296492
Slco1a5	4.04789

Lnpep	-7.945842
Ysk4	-11.651991
Slc5a1	9.234197
Saa3	26.291622
4833426H15Rik	-3.0473611
1700010A17Rik	-27.83073
1700094D03Rik	-25.349436
Enpp4	2.9199724
4933427G17Rik	-5.886851
D330014H01Rik	-74.64089
C86987	-10.863409
Lincr	45.80014
Lincr	61.500526
Sdcbp2	5.7782617
C230080I20Rik	-4.917731
Ch25h	4.627919
B230328G18Rik	-1.12808
Plxdc1	-3.5408723
Cte1	-20.435545
1700007G11Rik	-63.560345
6330406P08Rik	-6.3480644
B230396O12Rik	-14.911249
Dnaic1	-61.12469
1600029I14Rik	-21.598225
Sult1d1	-33.671505
Mccc2	-9.44772
Tmem20	-3.6095567
Cxcl16	7.8353767
Xylb	-14.6778345
AU021034	-31.521854
Sprr2e	123.3557
D11Ertd686e	-87.69467
BC004044	6.8603363
BC004044	13.766559
Dgat2	4.7578716
2410116G06Rik	-6.0053563
Plxnb3	-33.048252
LOC224813	-5.250548
Slc27a2	-3.3052275
LOC381284	-19.231024
1700026D08Rik	-30.082954
Hey1	-4.37703
LOC237891	-6.4645414

Mdm1	-20.532671
Syt5	-8.666486
0610009A07Rik	-26.136206
Clic4	5.730517
Ccne1	8.374979
1700018O18Rik	28.608704
9130024O20Rik	14.546422
Dfna5h	24.451979
LOC381050	14.717464
Wfdc13	-6.902324
BC051019	-22.941721
Tsga14	-15.265143
Rwdd2	-6.1151185
Rab4a	-4.1549053
Cxcl2	135.70998
C130081G24	4.4336486
Mak	-4.7741446
Tnfaip3	10.568073
4732452L12Rik	9.553486
BC013672	28.062946
Sult1a1	-12.560923
4931407K02Rik	-19.263119
Slfn4	9.825907
Nos3	-4.1788535
Il17c	264.16556
Lrriq2	-3.8740726
1700034E13Rik	-8.3601465
Ccna1	-51.234467
Fxyd2	-107.00877
Tcp11	-4.1918936
Wdr31	-22.951315
BC023105	35.56434
C230080E09Rik	-3.6145625
Irg1	507.68845
Irg1	390.2455
9130008F23Rik	7.797466
LOC195357	166.8272
1110007F12Rik	2.7291656
Fbxl13	-41.35467
Fbxl13	-18.400396
Ly64	3.6209624
Ly64	3.5992072
Dio1	-15.182323

Calcb	12.612391
LOC381736	-72.9035
Osmr	9.874561
Nos2	10.429437
Tnip1	6.3659554
LOC381245	131.37732
Casp4	8.567122
AU020206	13.60617
LOC382044	-12.625392
Slc2a6	130.07884
Il1a	37.127827
Il1a	48.53383
scl0002073.1_13	21.427965
Tln2	-4.1522803
0610037B23Rik	22.718325
Pglyrp4	34.590633
Nppb	12.309643
Psors1c2	75.07662
4430402I18Rik	-1.4967674
LOC384001	-1.6010369
AY078069	12.758204
BC036564	-52.573895
Ctse	-2.214388
Stxbp4	-5.7624807
Frk	2.9573557
LOC381270	-12.905317
Palm	-11.56739
scl000956.1_130	17.64839
Pglyrp4	74.39592
Slfn1	594.29346
M32486	-3.1736796
LOC240921	15.022007
Pdcd1lg1	23.670366
2510003B16Rik	-1.7940722
P2ry2	3.858005
1600029D21Rik	7.5845146
Hap1	6.7656994
0610007L01Rik	13.443201
Aifm3	-1.7380589
9930020N01Rik	15.108525
Ccdc39	-52.62793
Ccdc39	-11.084942
4930404N11Rik	-12.427151

Mad	14.658037
Mad	12.003484
Tst	-4.7814155
9930032O22Rik	3.6618903
4933428D01Rik	-95.88333
Pemt	4.6793065
Muc4	9.326261
Muc4	9.113364
Nln	-5.582543
Dnali1	-3.5106113
S100a8	3.9939768
4930504H06Rik	-4.5453143
Ptp4a2	2.423661
Cd40	189.85751
Ccdc96	-18.526485
3110023G01Rik	-9.711737
Aldh3a1	-3.4343762
Tnfaip2	44.811302
Tnfaip2	30.566004
Tnfaip2	30.717188
Slc7a4	-9.228412
Plat	6.908556
Fmo5	-9.574292
Snai3	5.6178327
Cbr3	-5.013678
2310014L17Rik	13.174543
6430537H07Rik	-52.213806
Chia	-35.650204
Indo	28.135294
Vav3	1.2001798
Tmem2	2.3640537
3300002A11Rik	-203.79768
Nek11	-13.514322
Stra6	10.800038
Plk3	6.5460773
Cd44	2.9366689
Cd44	2.809089
Stc2	-1.5616426
5330439J01Rik	-11.656634
Adam8	4.101046
Adam8	4.7410054
1110017D15Rik	-15.635389
1110017D15Rik	-14.668147

38047	-24.80307
Tnfrsf5	93.69475
3830408G10Rik	12.793762
5730508B09Rik	26.740488
Cry2	-3.788241
Speer4c	-26.022442
Gzmm	-4.2533712
A530065I17Rik	4.8945932
D15Wsu169e	-6.6967854
Hs3st1	6.0210423
Tnfsf10	41.742878
LOC381036	-46.774548
Dusp13	25.177153
Dusp13	12.620694
Ly6g5b	6.9437537
Eml1	-25.999874
Pccb	-9.281327
LOC381697	-3.17976
Lbh	-3.0362144
Cd59a	-5.1362534
3110032G18Rik	-4.442849
LOC328316	17.925676
Tnf	100.95429
Slc9a2	25.362368
Serpina9	1.2381666
Mogat1	-5.203873
1700003M02Rik	-25.30287
4930526H21Rik	-18.165352
4921511C04Rik	-10.323531
1700086L19Rik	-4.2145586
Steap	-1.4772114
4831415H04Rik	5.3691263
LOC195359	36.639946
Rbpsuh	8.222415
AI875199	-4.415329
BC007180	-3.6139836
Ldhd	-3.2405431
Gm1574	-78.98469
Gm1574	-31.619984
Pla1a	35.910603
LOC382447	1.9882851
Ttc29	-96.75366
Tpd52l1	-5.0660233

Tpd52l1	-5.058146
LOC381625	-5.017646
4930430E16Rik	-9.509059
LOC269531	-19.910957
Gbp5	79.894356
Sema7a	25.952602
Sema7a	66.42088
Nat5	10.944688
Smpdl3b	14.081125
Tff1	19.382963
Cldn10	-11.482289
Tsnaxip1	-86.01117
Tsnaxip1	-46.561455
2410004P03Rik	-17.128096
Pml	24.989002
B230373P09Rik	-85.82554
Ncoa2	4.969075
2510015F01Rik	-4.8195868
4932443I19Rik	-45.487377
scl00319622.1_241	4.8749294
1110049B09Rik	-12.393307
Dusp4	8.14277
Dusp4	53.133057
4930403G18Rik	-2.6593335
Gsta2	-6.8854537
2610041P08Rik	10.766775
Cldn10	-49.152927
BC094916	9.565945
1700003E16Rik	-56.22664
Slc25a18	-26.8294
A530050E01Rik	48.088715
Fmo3	-7.77887
Tomm70a	1.3728225
Rps6ka5	5.6027327
Ifitm1	22.51026
9530077C05Rik	-21.402067

Table 4 List of genes differentially expressed by co-exposed AECs compared to LPS or IAV exposure alone in Figure 16

Table 5 List of genes differentially expressed in co-exposed AECs compared to *S. pneumoniae* or IAV exposed alone in Figure 17

Symbol	FC ([x31+D39] vs. [mock])
Rnase1	-4.005454
Spag8	-9.05239
LOC381546	-3.2360406
LOC380658	-3.1613936
Adam7	19.34134
LOC381916	-8.504549
Irgm	9.037786
5730410E15Rik	-7.926718
Cxcl1	5.8797297
Ccl4	231.5049
Ccl4	28.141367
4833431D13Rik	-2.8066792
Casc1	-28.939758
LOC381260	-28.315615
Ak7	-5.942505
Acta1	-14.186584
LOC381165	-12.846548
scl0001045.1_6	-11.609933
Arrb1	-1.278096
4831417L10	-24.942825
Cxcl2	10.684248
LOC381336	-6.008809
scl0003377.1_167	-3.7938497
D4Bwg1540e	-4.565399
4930534B04Rik	-10.664519
Ttc18	-14.206028
Slc7a9	1.1069247
2900090M10Rik	-4.2518177
4931433A13Rik	-27.193962
1700067I02Rik	-23.424515
B230396O12Rik	-4.514424
4930579J09Rik	-4.5954247
1110020C03Rik	-7.340877
4933429I20Rik	-8.61118
Slpi	3.0236628
LOC381362	-5.5322275
C330001K17Rik	-7.423574
Ii	26.057446
Hs3st6	-1.9617084
4930533K18Rik	-8.717638

Zfp474	-4.7618046
A530060O05Rik	45.8859
Lnpep	-5.507354
Ii	14.868839
Ii	15.477973
Lincr	44.404655
Ccdc11	-2.0554867
Lnx1	16.161211
2410116G06Rik	-3.432547
Klk11	-2.4229279
Kcnq1	-3.7446623
Zp3	-5.6077
Vnn3	5.5073066
Vnn3	5.0325937
Cxcl2	12.230791
4931407K02Rik	-11.487261
Bicc1	-12.917514
Ubxd3	-3.5025134
BC025206	-3.1898446
Uox	-9.287806
Traf1	12.486707
scl0002073.1_13	8.048939
2410003A14Rik	-8.605818
Cxcl10	72.49143
Bphl	-3.0654972
Cd40	68.38418
Chia	-4.1731677
BC018465	15.089908
Tnfrsf5	18.104614
LOC226356	-33.549065
A730062O07Rik	-2.417968
Emid2	-8.197315
Ttc29	-8.052639
Abca8	-6.130264
3830422K02Rik	14.080281
4933437K13Rik	-3.1275494
4921537F17Rik	-4.2194486
A430083B19Rik	-15.520043
1700003E16Rik	-5.9079256
A530050E01Rik	17.887083

Table 5 List of genes differentially expressed in co-exposed AECS compared to *S. pneumoniae* or IAV exposure alone in Figure 17

Table 6 List of genes differentially expressed in co-infected whole lungs compared to *S. pneumoniae* or IAV infected alone in Figure 18

Symbol	FC ([5days + 48 hrs-X31+D39] vs. [5days + 48 hrs-PBS+PBS])
Aldh1a3	9.2532625
Gpr4	-2.4762802
Stfa2	14.25607
Peg3	-4.241927
Ccl1	11.8544445
LOC381287	23.939367
1700073K01Rik	-5.651048
Spic	7.586558
Hr	-3.8824863
Paqr6	-2.2973847
Cnfn	7.1827416
G630023A01Rik	-6.286888
Thrsp	-3.0482879
Gm614	4.396164
E230025N21Rik	-4.683217
E030040J04Rik	3.563552
D230040N21Rik	-1.9805455
1600013E24Rik	-2.841649
Setdb2	5.626637
Ldb3	-4.8544188
Clk3	3.1310713
1700111D19Rik	-2.8481288
Fech	-4.291375
Ftsj	-4.265552
Mx2	14.083483
G7e-pending	4.5161843
Ell3	2.934281
C330016K18Rik	-2.366802
A530023O14Rik	19.344511
A530023O14Rik	20.370556
4933407I18Rik	-11.14213
6430514L14Rik	-5.098839
Il18rap	8.825821
4933439C10Rik	-4.0925436
LOC328833	5.8336625
Mtap4	1.5323141
Slc7a11	18.059122

Slc7a11	14.081731
1300013B24Rik	2.2533054
Gsta1	-13.307004
Daam1	-4.749987
Tlr9	14.519885
Hsd11b2	5.63653
Hist1h2be	-3.6725256
4932425I24Rik	-4.282527
E430024F02Rik	6.1306915
Atp7b	-3.733271
Tnf	48.616432
G630009D10Rik	-3.1447508
LOC386545	16.019907
Erdr1	-6.245958
Rbp7	-2.569078
LOC232875	-4.863572
Stfa1	5.7176332
Stfa1	18.351698
Cxcl1	10.235682
0710008A13Rik	4.2041783
Cyp2e1	-4.951545
Nr1d1	-5.9089603
scl000034.1_162_REVCOMP	3.1244426
LOC382154	4.3704066
Krt1-13	10.807907
D7Bwg0611e	6.2388325
Ccl4	107.02748
A930013B19Rik	-4.1657777
scl0003168.1_6	9.344341
Gpr87	9.996748
LOC278666	30.618946
Zfpn1a1	9.0267725
EG433016	9.802567
Irs3	-7.2490907
IGKV8-21_Y15982_Ig_kappa_ variable_8-21_114	4.76241
Igfl3	5.1069446
LOC212718	5.752527
Fbln1	-5.9795547
9030205A07Rik	-13.992411
9830169C18Rik	-6.028699
LOC381879	8.650268
Krt2-6b	4.0608325

Acta1	45.53405
LOC381165	-4.3269534
Thy1	32.916348
Agpt	-10.409776
Dixdc1	-6.4854984
Aspn	-3.690544
LOC383860	-2.0231004
Olfr56	7.0751095
Pstpip2	13.208218
Kcna6	-8.368392
LOC278795	-2.5226665
LOC213439	15.658827
2610103H04Rik	3.8096554
Ifitm6	5.9908385
Ifnb1	41.085762
Stx11	6.9277024
Ccl3	87.47929
Mup2	2.3990302
Hspa1a	-2.948982
9430028F23Rik	8.407418
Cyp3a13	-1.9303551
Ambp	19.018637
Cyp2a4	-21.613972
Tff2	-60.54589
scl000078.1_211_REVCOMP	2.3224747
Pbp2	2.0175982
Mov10	3.784699
9030624G23Rik	4.0423946
Serpinb2	40.018063
1700023L04Rik	-7.0663724
Il8rb	4.689686
Gp2	-10.0725355
Prok2	5.0479474
Cxcl2	46.488182
2010002M12Rik	4.6770477
AI324046	28.00435
Vipr2	-2.7546325
2310003M01Rik	-3.085362
Phf11	5.9159303
Lhb	-10.715599
Slc7a11	6.443861
Lcp2	9.1721525
Il1rn	23.660025

Il1rn	4.5094924
LOC386508	20.890017
Grm6	2.2812102
Car3	-4.680913
Cspg2	4.072794
D630039M01Rik	-10.237084
Por	-6.7705603
Gzmk	262.37027
Slc39a12	-4.369838
Serpinb12	4.9955826
scl000085.1_5_REVCOMP	5.178832
Hif1a	4.8273635
BC018222	-3.2216098
Gstm6	-2.6782017
Csf2rb1	6.1862516
A430079P20Rik	-1.8763704
Cxcl10	600.971
C630002C17Rik	5.1121655
Dner	-9.2629
Pdzk3	-4.710562
Il10	64.81515
Awp1-pending	6.92514
D630040I23Rik	-1.8495762
2310003D02Rik	-2.649386
Lamc3	-5.04383
LOC381948	6.6153135
Gria1	-4.6646733
Tgtp	3.6317859
Slfn10	6.5077624
Il27	13.790844
Pkib	6.904138
B930018B01	-8.343017
Mb	-8.1575
Rptn	-3.3160033
Folh1	-21.115673
Krt1-16	3.6016574
Nptx1	-3.2138197
Hist1h3h	3.0532596
Avpr1a	1.3829854
Gprasp2	-11.598816
Mapt	-5.0617537
Clec5a	19.792244
A330030K22Rik	-2.407602

2310007F04Rik	5.5213084
IL1RA	123.79579
2610301F02Rik	-7.2090883
Osbpl1a	-2.8101897
Clecsf8	7.774864
BC067047	2.6944954
U46068	-32.76065
Oas3	4.301259
LOC228003	-3.5261433
Trim6	5.434816
A830094I09Rik	-4.0341988
4932415A06Rik	-11.353266
U46068	-25.698349
Gpr84	142.95872
Gpr84	59.014965
Slc6a4	-10.887822
Pamci	-6.349332
1700111A04Rik	-2.7490196
Lmnb1	7.577452
LOC386169	7.31161
Mlp	6.226706
Mlp	4.034599
Mlp	4.269826
E030025D05Rik	-9.970869
Casq2	-5.8209553
Car14	-4.271174
Ptafr	9.604911
Ccr8	10.151282
Hist1h3c	8.145356
LOC225609	-1.8443635
2300002G24Rik	17.832056
C130022C01Rik	-4.956253
9430028I06Rik	6.59567
D830007F02Rik	-4.5564327
1810007P19Rik	-3.8690012
LOC382177	17.124071
Dmn	-2.6123834
LOC239770	5.655685
Clca3	-2.5009844
Clca3	-6.8066072
A330042I05Rik	-7.794612
Ccrl1	-7.520608
2610302F08Rik	1.7147381

4632423N09Rik	-2.263005
Slc16a5	-5.0342126
LOC268885	7.1519656
Cxcl2	35.851307
D10Bwg0791e	-3.71564
Cyp2a5	-13.809622
Cyp2a5	-14.241847
Cyp2a5	-17.870626
C030003H22Rik	-4.818498
Pcdh20	-1.8066928
BC013672	20.122505
Smad7	-5.2284126
4931407K02Rik	-6.5050516
Slfn4	38.502865
Stfa3	23.420675
Nudt12	-3.1915348
Stfa1	17.068232
LOC381113	-4.6668243
scl000073.1_22_REVCOMP	7.5612593
scl0003154.1_1	4.8555236
2410076I21Rik	-4.182957
Ptx3	26.08214
Ptx3	52.39555
EG408196	18.178535
Irg1	221.22397
Irg1	774.8814
Aass	-2.4967082
Cd226	18.478956
2900079J23Rik	-4.218969
Trem3	4.121832
E130209G04Rik	-2.383037
Mlh3	1.0759473
LOC214531	-2.5398877
Bcl11b	10.718304
Cd14	2.9737172
TRAV6-1_AF259071_T_cell_receptor_alpha_variable_6-1_5	6.254975
LOC382866	-3.3937132
Calml3	-2.6970205
Slc7a11	15.205605
E330039G21Rik	-7.6393137
4930438O05Rik	-4.142868

Nos2	8.427788
9130221D24Rik	-4.8061395
Slc2a6	9.100533
9430014F16Rik	11.653433
Il1a	8.436582
Il1a	8.671223
scl0002073.1_13	13.485202
4632424B03Rik	5.787881
2300003P22Rik	16.990446
A630059M09Rik	6.741285
Sct	18.352911
9930115F03Rik	-8.229764
Pcdh7	-3.8082902
scl0001330.1_10	-3.6544385
2900074L10Rik	13.055641
Lep	-1.241542
Mpp2	-4.273582
Gpr109a	11.242366
G430091H17Rik	19.282732
Ear11	7.114176
4933432B09Rik	-4.10112
Syn3	4.8498545
LOC242805	-1.8072358
Fgf18	-4.46005
Ckm	-12.961521
A130002I06Rik	17.130575
Aim1l	-2.9392996
scl0003476.1_2802	-1.9170756
2810474C18Rik	4.0068674
Il1b	10.79308
Mad	7.5480456
Mad	21.078764
Vcan	4.748157
Cxcl10	386.2294
A030006P16Rik	-14.371817
Ctla4	60.410725
9330175E14Rik	2.7309005
Txlnb	-2.91859
G630052H11Rik	-3.1527958
2900093B09Rik	-5.905076
Sntb1	-1.1454355
Oasl1	69.75825
Oasl1	51.018208

BC057371	-2.2531562
Myl3	-3.5659628
scl000959.1_2	-2.207537
Ifng	142.51643
Ifng	45.64189
Mrgpra2	15.591386
1700012B09Rik	-5.395898
Clecsf9	29.983204
Clecsf9	15.015002
Hmox1	7.743844
Jam3	-4.479632
scl00009.1_36_REVCOMP	16.916739
Klra15	1.5972623
1700094J05Rik	2.2243679
Foxd4	5.5384827
Cd207	-12.154116
Trem1	6.071018
Fcrla	-1.3692479
Actb	5.7753654
Actb	4.6799197
Mmp8	6.8417115
Ank1	-3.3346198
Ank1	-3.3601944
Alpk3	-2.58638
A530017D12Rik	2.6464221
Ccl24	9.937703
Hcapg-pending	4.511475
E030003B04Rik	-2.6439447
Phlda2	-3.7232912
Syn3	-3.277085
Kcnf1	-2.6912522
Slc28a2	61.83259
Cd69	8.470249
A430056A10Rik	4.6036096
AF085738	6.0249553
C530008M17Rik	-10.654587
Tnf	74.86471
1300013J15Rik	-2.2643158
Bsg	-8.07679
Bsg	-5.726946
Serpina9	-3.391256
Scgb3a1	-47.363407
C730016G14Rik	-6.6665125

Edil3	-4.0142097
2700033K02Rik	-4.138234
2810451K12Rik	-6.4135146
Gm1574	-7.906493
mt-Nd5	12.727218
Expi	10.941179
Scgb3a1	-88.602585
Spa17	-2.6221395
Hspa1b	-3.8559456
A630012P03Rik	5.3480573
Vstm2	-6.946604
Usmg3	5.440463
Igf2bp3	3.678903
A830026L17Rik	-4.6222897
LOC386298	-6.8312926
Pkp1	4.66488
1700019G06Rik	5.820305
4930553M18Rik	2.7334979
1110012N22Rik	-2.533811
A530050D06Rik	-2.1718853
LOC381146	-7.3547177
Gprk2l	-5.474133
Prom2	-4.3783746
Tcf1	2.5104222
BC094916	12.335565
Mrgpra7	5.5885677
Itgbl1	3.0382054

Table 6 List of genes differentially expressed in co-infected whole lungs compared to *S. pneumoniae* or IAV alone infected alone in Figure 18

Table 7 The gene list from the cell death and survival pathway in Figure 42D

Symbol	FC ([Alpha] vs. [Media])	FC ([beta] vs. [Media])	FC ([lamda] vs. [Media])
Hap1	1.5368242	1.6429482	4.650505
Psmb10	1.3218682	1.5655625	4.461529
Psmb10	1.3302051	1.4910861	4.33169
Nt5c3	1.0158954	-1.0787896	2.4042892
Irf7	46.627216	146.663	828.19336
Muc1	1.192938	1.2522265	2.0618737
Muc1	1.139818	1.1713599	2.0833116
Fgfr1	-1.1365047	7.1685767	-1.0663099
Irgm	3.205464	4.28573	14.825324
Gsta1	-1.1697929	-1.7688241	-4.289652
Gsta1	-1.2170107	-1.8316518	-3.6169176
Gsta1	-1.1584047	-1.6666508	-5.4789934
Tnfrsf11b	1.2631515	1.4013133	6.1671166
Irf1	1.1271989	1.2133054	2.2168913
Irf1	1.0988456	1.1622128	2.088025
Pml	1.2538916	1.2722906	2.503706
Tdrd7	1.3670524	1.3958756	2.5815835
Ifit2	1.2089369	2.0138652	35.108536
Ifit2	1.2698854	2.7552	42.715237
B2m	2.8235974	4.727805	9.850022
B2m	3.009347	5.113851	10.285195
Txnip	1.0627877	1.1859338	2.4460237
Isg20	1.2627181	1.9371066	6.329873
Scd1	-1.16213	-1.370751	-2.3313024
Gadd45g	1.3798066	1.8352783	2.6689386
Gadd45g	1.5746205	1.9802735	3.8099658
Trim21	1.9199371	2.6484532	10.456548
Lgals3bp	5.980614	8.171425	21.214617
Lmo2	1.1730537	-1.0117885	7.1917257
Casp1	1.2345303	1.1968346	2.6525166
Optn	1.4300839	1.3557348	2.56553
Tapbp	1.3910825	1.4895842	4.12192
Parp14	3.755458	5.3035007	17.907188
Sort1	-1.0998355	-1.3885114	-2.506992
Tap1	3.341829	4.5298557	20.053694
Adar	2.1189837	3.9119709	10.668044
Cd47	1.2327391	1.1701299	3.4737177
Sort1	-1.2232996	-1.4289604	-2.5285811
Plcd1	-1.4457713	-1.7698541	-3.9379032
H2-T23	1.5179851	2.0125878	8.31526

H2-T23	1.6318599	2.3127959	6.9104276
H2-T23	1.5618751	2.148976	8.652974
Il15	1.3950137	1.6807132	3.3993754
Oas3	8.389993	14.457958	66.79426
H2-K1	1.4208133	2.3953323	7.3155456
Usp18	3.6112924	5.7998853	19.54635
Usp18	3.401401	4.730425	14.133283
Usp18	3.7943451	6.0265775	27.15867
Ddit3	-1.0515045	1.2992167	5.2128167
Ddit3	1.6529723	1.5567713	6.9476175
Psmb8	2.5984392	4.568623	28.468575
Psmb8	1.8362747	3.207279	25.738182
Igfbp5	-1.1119783	-1.1305896	-2.5272732
Igfbp5	-1.0542079	-1.1112237	-2.251628
Clic4	1.4665444	1.5921469	2.3563085
Cyr61	1.2197021	1.4718664	2.167132
Mlkl	1.2147489	1.2303972	2.9012816
Stat2	3.9967399	7.6267147	37.49118
Tgm1	-1.299958	-1.5127536	-2.1610565
Daxx	1.2571676	1.3861653	3.744458
Igfbp5	-1.1425205	-1.2025253	-2.462872
Lgals8	1.1556364	1.3102657	2.407126
Lgals8	1.2180737	1.3407359	2.6079981
Trex2	1.0123321	-1.071811	2.3668616
Casp4	1.3045112	1.5389134	4.3948336
Il1a	1.0248451	-1.2250439	2.3398027
Txnip	1.0614768	1.1434577	2.3508062
Rnf31	2.1212373	2.3792245	4.457319
Rbm43	1.1655492	1.2214872	2.528525
Adar	2.1739302	2.9827142	8.134632
Adar	2.0044742	2.392418	3.3280604
Cdc42ep3	1.1717758	1.2427976	2.113384
Oas1b	-1.1009653	5.3312135	73.88632
Irf5	1.2627467	1.0022689	4.130327
H2-M3	1.4727287	1.3134012	4.79143
Btc	1.2036638	1.2286314	2.6748407
Ifit3	10.296514	47.546497	934.8526
Ifit3	9.554748	46.45387	1523.5782
Ifit3	11.627982	51.944935	1667.002
Trex1	1.194127	1.795586	9.617526
Trex1	1.6882477	2.0657175	14.095984
Aldh3a1	-1.2339481	-1.6135081	-3.7445908
Tnfaip2	-1.2731305	-1.6087726	-2.8936818

Gch1	1.146738	1.1844139	2.6152468
Gch1	1.1457549	-1.1357046	2.2476487
Plat	1.1357344	1.2480022	2.1409032
Lama1	-1.1629443	-1.3475317	-2.319883
Gnpnat1	-9.133604	-10.3266535	-9.809605
Atf3	-1.0624293	1.2637888	2.6643963
Pcsk9	-1.0742208	-1.3455986	-2.3364913
H2-K1	1.1868508	3.2084458	13.557327
Ephb6	-9.782708	-9.662275	-8.637292
Tap2	1.4973706	1.6387764	5.655904
Gas6	-1.116394	-1.7596534	-2.2108812
Plac8	1.0006844	1.0887637	2.5553946
Prkcdbp	1.065209	1.3301984	3.43014
Prkcdbp	1.0456362	1.3674774	4.0843987
Stat1	2.7910256	4.0751166	16.041254
Stat1	3.000436	3.8189561	17.763748
Sema7a	1.0215431	1.3746121	2.6188538
Spink2	1.9964968	2.2641604	6.470621
Mt1	-1.1559778	-1.2769563	-2.058757
Timp3	1.3815713	1.7367173	3.3761735
Gsta2	-1.0847636	-1.5502074	-3.1348186

Table 7 The gene list from the cell death and survival pathway in Figure 42D

Reference List

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